

5 **STABILIZED BIOACTIVE PEPTIDES AND METHODS OF**
 IDENTIFICATION, SYNTHESIS AND USE

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Background of the Invention

 Bioactive peptides are small peptides that elicit a biological activity.
Since the discovery of secretin in 1902, over 500 of these peptides which
15 average 20 amino acids in size have been identified and characterized. They
have been isolated from a variety of systems, exhibit a wide range of actions,
and have been utilized as therapeutic agents in the field of medicine and as
diagnostic tools in both basic and applied research. Tables 1 and 2 list some of
the best known bioactive peptides.

Table 1: Bioactive peptides utilized in medicine

Name	Isolated From	Size In Amino Acids	Therapeutic Use
Angiotensin II	Human Plasma	8	Vasoconstrictor
Bradykinin	Human Plasma	9	Vasodilator
Caerulein	Frog Skin	10	Choleretic Agent
Calcitonin	Human Parathyroid Gland	32	Calcium Regulator
Cholecystokinin	Porcine Intestine	33	Choleretic Agent
Corticotropin	Porcine Pituitary Gland	39	Hormone
Eledoisin	Octopod Venom	11	Hypotensive Agent
Gastrin	Porcine Stomach	17	Gastric Activator
Glucagon	Porcine Pancreas	29	Antidiabetic Agent
Gramicidin D	<i>Bacillus brevis</i> Bacteria	11	Antibacterial Agent
Insulin	Canine Pancreas		Antidiabetic Agent
Insulin A		21	
Insulin B		30	
Kallidin	Human Plasma	10	Vasodilator
Luteinizing Hormone- Releasing Factor	Bovine Hypothalamus	10	Hormone Stimulator
Melittin	Bee Venom	26	Antirheumatic Agent
Oxytocin	Bovine Pituitary Gland	9	Oxytocic Agent
Secretin	Canine Intestine	27	Hormone
Sermorelin	Human Pancreas	29	Hormone Stimulator
Somatostatin	Bovine Hypothalamus	14	Hormone Inhibitor
Vasopressin	Bovine Pituitary Gland	9	Antidiuretic Agent

Table 2: Bioactive peptides utilized in applied research

Name	Isolated From	Size In Amino Acids	Biological Activity
Atrial Natriuretic Peptide	Rat Atria	28	Natriuretic Agent
Bombesin	Frog Skin	14	Gastric Activator
Conantokin G	Snail Venom	17	Neurotransmitter
Conotoxin GI	Snail Venom	13	Neuromuscular Inhibitor
Defensin HNP-1	Human Neutrophils	30	Antimicrobial Agent
Delta Sleep-Inducing Peptide	Rabbit Brain	9	Neurological Affecter
Dermaseptin	Frog Skin	34	Antimicrobial Agent
Dynorphin	Porcine Brain	17	Neurotransmitter
EETI II	<i>Ecballium elaterium</i> seeds	29	Protease Inhibitor
Endorphin	Human Brain	30	Neurotransmitter
Enkephalin	Human Brain	5	Neurotransmitter
Histatin 5	Human Saliva	24	Antibacterial Agent
Mastoparan	<i>Vespid</i> Wasps	14	Mast Cell Degranulator
Magainin I	Frog Skin	23	Antimicrobial Agent
Melanocyte Stimulating Hormone	Porcine Pituitary Gland	13	Hormone Stimulator
Motilin	Canine Intestine	22	Gastric Activator
Neurotensin	Bovine Brain	13	Neurotransmitter
Physalaemin	Frog Skin	11	Hypotensive Agent
Substance P	Horse Intestine	11	Vasodilator
Vasoactive Intestinal Peptide	Porcine Intestine	28	Hormone

Where the mode of action of these peptides has been determined, it has been found to be due to the interaction of the bioactive peptide with a specific protein target. In most of the cases, the bioactive peptide acts by binding to and inactivating its protein target with extremely high specificities. Binding constants of these peptides for their protein targets typically have been determined to be in the nanomolar (nM, 10^{-9} M) range with binding constants as high as 10^{-12} M (picomolar range) having been reported. Table 3 shows target proteins inactivated by several different bioactive peptides as well as the binding constants associated with binding thereto.

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Table 3: Binding constants of bioactive peptides

Bioactive Peptide	Size in Amino Acids	Inhibited Protein	Binding Constant
α -Conotoxin GIA	15	Nicotinic Acetylcholine Receptor	1.0×10^{-9} M
EETI II	29	Trypsin	1.0×10^{-12} M
H2 (7-15)	8	HSV Ribonucleotide Reductase	3.6×10^{-5} M
Histatin 5	24	Bacteroides gingivalis Protease	5.5×10^{-8} M
Melittin	26	Calmodulin	3.0×10^{-9} M
Myotoxin (29-42)	14	ATPase	1.9×10^{-5} M
Neurotensin	13	Ni Regulatory Protein	5.6×10^{-11} M
Pituitary Adenylate Cyclase Activating Polypeptide	38	Calmodulin	1.5×10^{-8} M
PKI (5-24)	20	cAMP-Dependent Protein Kinase	2.3×10^{-9} M
SCP (153-180)	27	Calpain	3.0×10^{-8} M
Secretin	27	HSR G Protein	3.2×10^{-9} M
Vasoactive Intestinal Peptide	28	GPRN1 G Protein	2.5×10^{-9} M

Recently, there has been an increasing interest in employing synthetically derived bioactive peptides as novel pharmaceutical agents due to the impressive ability of the naturally occurring peptides to bind to and inhibit specific protein targets. Synthetically derived peptides could be useful in the development of
5 new antibacterial, antiviral, and anticancer agents. Examples of synthetically derived antibacterial or antiviral peptide agents would be those capable of binding to and preventing bacterial or viral surface proteins from interacting with their host cell receptors, or preventing the action of specific toxin or protease proteins. Examples of anticancer agents would include synthetically derived
10 peptides that could bind to and prevent the action of specific oncogenic proteins.

To date, novel bioactive peptides have been engineered through the use of two different *in vitro* approaches. The first approach produces candidate peptides by chemically synthesizing a randomized library of 6-10 amino acid peptides (J. Eichler et al., Med. Res. Rev. **15**:481-496 (1995); K. Lam,
15 Anticancer Drug Des. **12**:145-167 (1996); M. Lebl et al., Methods Enzymol. **289**:336-392 (1997)). In the second approach, candidate peptides are synthesized by cloning a randomized oligonucleotide library into a Ff filamentous phage gene, which allows peptides that are much larger in size to be expressed on the surface of the bacteriophage (H. Lowman, Ann. Rev. Biophys.
20 Biomol. Struct. **26**:401-424 (1997); G. Smith et al., et al. Meth. Enz. **217**:228-257 (1993)). To date, randomized peptide libraries up to 38 amino acids in length have been made, and longer peptides are likely achievable using this system. The peptide libraries that are produced using either of these strategies are then typically mixed with a preselected matrix-bound protein target.
25 Peptides that bind are eluted, and their sequences are determined. From this information new peptides are synthesized and their inhibitory properties are determined. This is a tedious process that only screens for one biological activity at a time.

Although these *in vitro* approaches show promise, the use of
30 synthetically derived peptides has not yet become a mainstay in the pharmaceutical industry. The primary obstacle remaining is that of peptide instability within the biological system of interest as evidenced by the unwanted degradation of potential peptide drugs by proteases and/or peptidases in the host

cells. There are three major classes of peptidases which can degrade larger peptides: amino and carboxy exopeptidases which act at either the amino or the carboxy terminal end of the peptide, respectively, and endopeptidases which act on an internal portion of the peptide. Aminopeptidases, carboxypeptidases, and endopeptidases have been identified in both prokaryotic and eukaryotic cells. Many of those that have been extensively characterized were found to function similarly in both cell types. Interestingly, in both prokaryotic and eukaryotic systems, many more aminopeptidases than carboxypeptidases have been identified to date.

Approaches used to address the problem of peptide degradation have included the use of D-amino acids or modified amino acids as opposed to the naturally occurring L-amino acids (e.g., J. Eichler et al., *Med Res Rev.* **15**:481-496 (1995); L. Sanders, *Eur. J. Drug Metabol. Pharmacokinetics* **15**: 95-102 (1990)), the use of cyclized peptides (e.g., R. Eggleton, et al., *Peptides* **18**:1431-1439 (1997)), and the development of enhanced delivery systems that prevent degradation of a peptide before it reaches its target in a patient (e.g., L. Wearley, *Crit. Rev. Ther. Drug Carrier Syst.* **8**: 331-394 (1991); L. Sanders, *Eur. J. Drug Metabol. Pharmacokinetics* **15**: 95-102 (1990)). Although these approaches for stabilizing peptides and thereby preventing their unwanted degradation in the biosystem of choice (e.g., a patient) are promising, there remains no way to routinely and reliably stabilize peptide drugs and drug candidates. Moreover, many of the existing stabilization and delivery methods cannot be directly utilized in the screening and development of novel useful bioactive peptides. A biological approach that would serve as both a method of stabilizing peptides and a method for identifying novel bioactive peptides would represent a much needed advance in the field of peptide drug development.

Summary of the Invention

The present invention provides an intracellular screening method for identifying novel bioactive peptides. A host cell is transformed with an expression vector comprising a tightly regulable control region operably linked to a nucleic acid sequence encoding a peptide. The transformed host cell is first grown under conditions that repress expression of the peptide and then, subsequently, expression of the peptide is induced. Phenotypic changes in the host cell upon expression of the peptide are indicative of bioactivity, and are evaluated. If, for example, expression of the peptide is accompanied by inhibition of host cell growth, the expressed peptide constitutes a bioactive peptide, in that it functions as an inhibitory peptide.

Intracellular identification of bioactive peptides can be advantageously carried out in a pathogenic microbial host cell. Bioactive peptides having antimicrobial activity are readily identified in a microbial host cell system. Further, the method can be carried out in a host cell that has not been modified to reduce or eliminate the expression of naturally expressed proteases or peptidases. When carried out in a host cell comprising proteases and peptides, the selection process of the invention is biased in favor of bioactive peptides that are protease- and peptidase-resistant.

The tightly regulable control region of the expression vector used to transform the host cell according to the invention is preferably derived from the wild-type *Escherichia coli* lac operon, and the transformed host cell preferably comprises an amount of Lac repressor protein effective to repress expression of the peptide during host cell growth under repressed conditions. To insure a sufficient amount of Lac repressor protein, the host cell can be transformed with a second vector the overproduces Lac repressor protein.

Optionally, the expression vector used to transform the host cell can be genetically engineered to encode a stabilized peptide that is resistant to peptidases and proteases. For example, the coding sequence can be designed to encode a stabilizing group at either or both of the peptide N-terminus or C-terminus. As another example, the coding sequence can be designed to encode a stabilizing motif such as an α -helix motif or an opposite charge ending motif, as

described below. The presence of a stabilizing group at a peptide terminus or a stabilizing motif can slow down the rate of intracellular degradation of the peptide.

The invention further provides a bioactive peptide having a first
5 stabilizing group comprising the N-terminus and a second stabilizing group comprising the C-terminus. Preferably, the first stabilizing group is selected from the group consisting of a small stable protein, Pro-, Pro-Pro-, Xaa-Pro- and Xaa-Pro-Pro-; and the second stabilizing group is selected from the group consisting of a small stable protein, -Pro, -Pro-Pro, -Pro-Xaa and -Pro-Pro-Xaa.
10 Suitable small stable proteins include Rop protein, glutathione sulfotransferase, thioredoxin, maltose binding protein, and glutathione reductase. In addition, the invention provides a bioactive peptide stabilized by an opposite charge ending motif, as described below. The bioactive peptide is preferably an antimicrobial peptide or a therapeutic peptide drug.

15 Also provided by the invention is a polypeptide that can be cleaved to yield a bioactive peptide having a stabilizing group at either or both of its N- and C-termini. The cleavable polypeptide accordingly comprises a chemical or enzymatic cleavage site either immediately preceding the N-terminus of the bioactive peptide or immediately following the C-terminus of the bioactive
20 peptide.

The invention further provides a fusion protein comprising a four-helix bundle protein, preferably the Rop protein, and a polypeptide. The four-helix bundle protein is positioned at either the N-terminus or the C-terminus of the fusion protein, and accordingly can be fused to either the N-terminus or the C-
25 terminus of the polypeptide.

The present invention also provides a method for using an antimicrobial peptide. An antimicrobial peptide is stabilized by linking a first stabilizing group to the N-terminus of an antimicrobial peptide, and, optionally, a second stabilizing group to the C-terminus of the antimicrobial peptide. Alternatively,
30 the antimicrobial peptide is stabilized by flanking the peptide sequence with an opposite charge ending motif, as described below. The resulting stabilized antimicrobial peptide is brought into contact with a microbe, preferably a pathogenic microbe, for example to inhibit the growth or toxicity of the microbe.

The invention also provides a method for treating a patient having a condition treatable with a peptide drug, comprising administering to the patient a stabilized peptide drug having at least one of a first stabilizing group comprising the N-terminus of the stabilized peptide drug and a second stabilizing group comprising the C-terminus of the stabilized peptide drug. Optionally, prior to administration of the stabilized peptide drug, the first stabilizing group is covalently linked to the N-terminus of a peptide drug, and the second stabilizing group is covalently linked to the C-terminus of the peptide drug to yield the stabilized peptide drug. Alternatively, the method comprises administering to the patient a peptide drug that has been stabilized by flanking the peptide sequence with an opposite charge ending motif, as described below.

Brief Description of the Drawings

Figure 1 shows the control region (SEQ ID NO:1) of the wild-type *lac* operon from the auxiliary operator O3 through the translational start of the *lacZ* gene. DNA binding sites include the operators O3 and O1 (both underlined), CAP (boxed), the -35 site (boxed), and the -10 site (boxed), while important RNA and protein sites include the LacI translation stop site (TGA), the +1 *lacZ* transcription start site, the Shine Dalgarno (SD) ribosome binding site for *lacZ*, and the LacZ translation start site (ATG).

Figure 2 is a map of plasmid pLAC11. The unique restriction sites and the base pair at which they cut are indicated. Other sites of interest are also shown, including Tet (98-1288), Rop (1931-2122), ori (2551-3138), Amp (3309-4169), and *lacPO* (4424-4536).

Figure 3 is a map of plasmid pLAC22. The unique restriction sites and the base pair at which they cut are indicated. Other sites of interest are also shown, including Tet (98-1288), Rop (1927-2118), ori (2547-3134), Amp (3305-4165), *lacIq* (4452-5536), and *lacPO* (5529-5641).

Figure 4 is a map of plasmid pLAC33. The unique restriction sites and the base pair at which they cut are indicated. Other sites of interest are also shown, including Tet (98-1288), ori (1746-2333), Amp (2504-3364), and *lacPO* (3619-3731).

Figure 5 shows the response of the pLAC11-*lacZ* construct (open circles) to varying amounts of isopropyl β -D-thiogalactoside (IPTG). A filled square indicates the β -galactosidase activity that was obtained when MG1655 or CSH27 cells were grown in rich media induced with 1 mM IPTG, while a filled diamond indicates the β -galactosidase activity that was obtained when MG1655 or CSH27 cells were grown in M9 minimal lactose media.

Figure 6 shows growth curves depicting the inhibitory effects of a two day inhibitor (pPep12) versus a one day inhibitor (pPep1). Data points for the control, pLAC11, for pPep1, and for pPep12, are indicated by squares, circles, and triangles, respectively.

Figure 7 is a map of the p-Rop(C) fusion vector. The unique restriction sites and the base pair at which they cut are indicated. Other sites of interest are also shown, including Rop (7-198), ori (627-1214), Amp (2245-1385), lacPO (2500-2612).

Figure 8 is a map of the p(N)Rop-fusion vector. The unique restriction sites and the base pair at which they cut are indicated. Other sites of interest are also shown; Rop (7-204), ori (266-853), Amp (1024-1884), lacPO (2139-2251).

Figure 9 illustrates a peptide (SEQ ID NO:2) having the opposite charge ending motif, wherein the amino and carboxy termini of the peptide are stabilized by the interactions of the opposite charge ending amino acids.

Detailed Description

The present invention represents a significant advance in the art of peptide drug development by allowing concurrent screening for peptide bioactivity and stability. Randomized recombinant peptides are screened for bioactivity in a tightly regulated inducible expression system, preferably derived from the wild-type *lac* operon, that permits essentially complete repression of peptide expression in the host cell. Subsequent induction of peptide expression can then be used to identify peptides that inhibit host cell growth or possess other bioactivities.

Intracellular screening of randomized peptides has many advantages over existing methods. Bioactivity is readily apparent, many diverse bioactivities can

be screened for simultaneously; very large numbers of peptides can be screened using easily generated peptide libraries, and the host cell, if desired, can be genetically manipulated to elucidate an affected protein target. Advantageously, randomized peptides can be screened in a host cell that is identical to or closely resembles the eventual target cell for antimicrobial applications. An additional and very important feature of this system is that selection is naturally biased in favor of peptides that are stable in an intracellular environment; i.e., that are resistant to proteases and peptidases. Fortuitously, bacterial peptidases are very similar to eukaryotic peptidases. Peptides that are stable in a bacterial host are thus likely to be stable in a eukaryotic cell as well, allowing bacterial cells to be used in initial screens to identify drugs that may eventually prove useful as human or animal therapeutics.

The invention is directed to the identification and use of bioactive peptides. A bioactive peptide is a peptide having a biological activity. The term "bioactivity" as used herein includes, but is not limited to, any type of interaction with another biomolecule, such as a protein, glycoprotein, carbohydrate, for example an oligosaccharide or polysaccharide, nucleotide, polynucleotide, fatty acid, hormone, enzyme, cofactor or the like, whether the interactions involve covalent or noncovalent binding. Bioactivity further includes interactions of any type with other cellular components or constituents including salts, ions, metals, nutrients, foreign or exogenous agents present in a cell such as viruses, phage and the like, for example binding, sequestration or transport-related interactions. Bioactivity of a peptide can be detected, for example, by observing phenotypic effects in a host cell in which it is expressed, or by performing an *in vitro* assay for a particular bioactivity, such as affinity binding to a target molecule, alteration of an enzymatic activity, or the like. Examples of bioactive peptides include antimicrobial peptides and peptide drugs. Antimicrobial peptides are peptides that adversely affect a microbe such as a bacterium, virus, protozoan, or the like. Antimicrobial peptides include, for example, inhibitory peptides that slow the growth of a microbe, microbiocidal peptides that are effective to kill a microbe (e.g., bacteriocidal and virocidal peptide drugs, sterilants, and disinfectants), and peptides effective to interfere with microbial reproduction, host toxicity, or the like. Peptide drugs for therapeutic use in

humans or other animals include, for example, antimicrobial peptides that are not prohibitively toxic to the patient, peptides designed to elicit, speed up, slow down, or prevent various metabolic processes in the host such as insulin, oxytocin, calcitonin, gastrin, somatostatin, anticancer peptides, and the like.

5 The term "peptide" as used herein refers to a plurality of amino acids joined together in a linear chain via peptide bonds. Accordingly, the term "peptide" as used herein includes a dipeptide, tripeptide, oligopeptide and polypeptide. A dipeptide contains two amino acids; a tripeptide contains three amino acids; and the term oligopeptide is typically used to describe peptides
10 having between 2 and about 50 or more amino acids. Peptides larger than about 50 are often referred to polypeptides or proteins. For purposes of the present invention, a "peptide" is not limited to any particular number of amino acids. Preferably, however, the peptide contains about 2 to about 50 amino acids, more preferably about 5 to about 40 amino acids, most preferably about 5 to about 20
15 amino acids.

 The library used to transform the host cell is formed by cloning a randomized, peptide-encoding oligonucleotide into a nucleic acid construct having a tightly regulable expression control region. An expression control region can be readily evaluated to determine whether it is "tightly regulable," as
20 the term is used herein, by bioassay in a host cell engineered to contain a mutant nonfunctional gene "X." Transforming the engineered host cell with an expression vector containing a tightly regulable expression control region operably linked to a cloned wild-type gene "X" will preserve the phenotype of the engineered host cell under repressed conditions. Under induced conditions,
25 however, the expression vector containing the tightly regulable expression control region that is operably linked to the cloned wild-type gene "X" will complement the mutant nonfunctional gene X to yield the wild-type phenotype. In other words, a host cell containing a null mutation which is transformed with a tightly regulable expression vector capable of expressing the chromosomally
30 inactivated gene will exhibit the null phenotype under repressed conditions; but when expression is induced the cell will exhibit a phenotype indistinguishable from the wild-type cell. It should be understood that the expression control region in the tightly regulable expression vector of the present invention can be

readily modified to produce higher levels of an encoded biopeptide, if desired (see, e.g., Example I, below). Such modification may unavoidably introduce some "leakiness" into expression control, resulting in a low level of peptide expression under repressed conditions.

5 In a preferred embodiment, the expression control region of the inducible expression vector is derived from the wild-type *E. coli lac* promoter/operator region. In a particularly preferred form, the expression vector contains a regulatory region that includes the auxiliary operator O₃, the CAP binding region, the -35 promoter site, the -10 promoter site, the operator O₁, the Shine-Dalgarno sequence for *lacZ*, and a spacer region between the end of the Shine-Dalgarno sequence and the ATG start of the *lacZ* coding sequence (see Fig. 1).

It is to be understood that variations in the wild-type nucleic acid sequence of the *lac* promoter/operator region can be tolerated in the expression control region of the preferred expression vector and are encompassed by the invention, provided that the expression control region remains tightly regulable as defined herein. For example, the -10 site of the wild-type *lac* operon (TATGTT) is weak compared to the bacterial consensus -10 site sequence TATAAT, sharing four out of six positions. It is contemplated that other comparably weak promoters are equally effective at the -10 site in the expression control region; a strong promoter is to be avoided in order to insure complete repression in the uninduced state. With respect to the -35 region, the sequence of the wild-type *lac* operon, TTTACA, is one base removed from the consensus -35 sequence TTGACA. It is contemplated that a tightly regulable *lac* operon-derived expression control region could be constructed using a weaker -35 sequence (i.e., one having less identity with the consensus -35 sequence) and a wild-type -10 sequence (TATAAT), yielding a weak promoter that needs the assistance of the CAP activator protein. Similarly, it is to be understood that the nucleic acid sequence of the CAP binding region can be altered as long as the CAP protein binds to it with essentially the same affinity.

30 The spacer region between the end of the Shine-Dalgarno sequence and the ATG start of the *lacZ* coding sequence is typically between about 5 and about 10 nucleotides in length, preferably about 5 to about 8 nucleotides in length, more preferably about 7-9 nucleotides in length. The most preferred composition and

length of the spacer region depends on the composition and length of Shine-Dalgarno sequence with which it is operably linked as well as the translation start codon employed (i.e., AUG, GUG, or UUG), and can be determined accordingly by one of skill in the art. Preferably the nucleotide composition of
5 the spacer region is "AT rich"; that is, it contains more A's and T's than it does G's and C's.

In a preferred embodiment of the method of the invention, the expression vector has the identifying characteristics of pLAC11 (ATCC No. 207108). More preferably, the expression vector is pLAC11 (ATCC No. 207108).

10 As used in the present invention, the term "vector" is to be broadly interpreted as including a plasmid, including an episome, a viral vector, a cosmid, or the like. A vector can be circular or linear, single-stranded or double-stranded, and can comprise RNA, DNA, or modifications and combinations thereof. Selection of a vector or plasmid backbone depends upon a variety of
15 characteristics desired in the resulting construct, such as selection marker(s), plasmid copy number, and the like. A nucleic acid sequence is "operably linked" to an expression control sequence in the regulatory region of a vector, such as a promoter, when the expression control sequence controls or regulates the transcription and/or the translation of that nucleic acid sequence. A nucleic
20 acid that is "operably linked" to an expression control sequence includes, for example, an appropriate start signal (e.g., ATG) at the beginning of the nucleic acid sequence to be expressed and a reading frame that permits expression of the nucleic acid sequence under control of the expression control sequence to yield production of the encoded peptide. The regulatory region of the expression
25 vector optionally includes a termination sequence, such as a codon for which there is no corresponding aminoacyl-tRNA, thus ending peptide synthesis. Typically, when the ribosome reaches a termination sequence or codon during translation of the mRNA, the polypeptide is released and the ribosome-mRNA-tRNA complex dissociates.

30 An expression vector optionally includes one or more selection or marker sequences, which typically encode an enzyme capable of inactivating a compound in the growth medium. The inclusion of a marker sequence can, for example, render the host cell resistant to an antibiotic, or it can confer a

compound-specific metabolic advantage on the host cell. Cells can be transformed with the expression vector using any convenient method known in the art, including chemical transformation, e.g., whereby cells are made competent by treatment with reagents such as CaCl_2 ; electroporation and other electrical techniques; microinjection and the like.

In embodiments of the method that make use of a tightly regulable expression system derived from the *lac* operon, the host cell is or has been genetically engineered or otherwise altered to contain a source of Lac repressor protein in excess of the amount produced in wild-type *E. coli*. A host cell that contains an excess source of Lac repressor protein is one that expresses an amount of Lac repressor protein sufficient to repress expression of the peptide under repressed conditions, i.e., in the absence of an inducing agent, such as isopropyl β -D-thiogalactoside (IPTG). Preferably, expression of Lac repressor protein is constitutive. For example, the host cell can be transformed with a second vector comprising a gene encoding Lac repressor protein, preferably *lacI*, more preferably *lacI^q*, to provide an excess source of Lac repressor protein in trans, i.e., extraneous to the tightly regulable expression vector. An episome can also serve as a trans source of Lac repressor. Another option for providing a trans source of Lac repressor protein is the host chromosome itself, which can be genetically engineered to express excess Lac repressor protein. Alternatively, a gene encoding Lac repressor protein can be included on the tightly regulable expression vector that contains the peptide-encoding oligonucleotide so that Lac repressor protein is provided in cis. The gene encoding the Lac repressor protein is preferably under the control of a constitutive promoter.

The invention is not intended to be limited in any way by the type of host cell used for screening. The host cell can be a prokaryotic or a eukaryotic cell. Preferred mammalian cells include human cells, of any tissue type, and can include cancer cells or hybridomas, without limitation. Preferred bacterial host cells include gram negative bacteria, such as *E. coli* and various *Salmonella* spp., and gram positive bacteria, such as bacteria from the genera *Staphylococcus*, *Streptococcus* and *Enterococcus*. Protozoan cells are also suitable host cells. In clear contrast to conventional recombinant protein expression systems, it is preferable that the host cell contains proteases and/or peptidases, since the

selection will, as a result, be advantageously biased in favor of peptides that are protease- and peptidase-resistant. More preferably, the host cell has not been modified, genetically or otherwise, to reduce or eliminate the expression of any naturally expressed proteases or peptidases. The host cell can be selected with a particular purpose in mind. For example, if it is desired to obtain peptide drugs specific to inhibit *Staphylococcus*, peptides can be advantageously expressed and screened in *Staphylococcus*.

There is, accordingly, tremendous potential for the application of this technology in the development of new antibacterial peptides useful to treat various pathogenic bacteria. Of particular interest are pathogenic *Staphylococci*, *Streptococci*, and *Enterococci*, which are the primary causes of nosocomial infections. Many of these strains are becoming increasingly drug-resistant at an alarming rate. The technology of the present invention can be practiced in a pathogenic host cell to isolate inhibitor peptides that specifically target the pathogenic strain of choice. Inhibitory peptides identified using pathogenic microbial host cells in accordance with the invention may have direct therapeutic utility; based on what is known about peptide import, it is very likely that small peptides are rapidly taken up by *Staphylococci*, *Streptococci*, and *Enterococci*. Once internalized, the inhibitory peptides identified according to the invention would be expected to inhibit the growth of the bacteria in question. It is therefore contemplated that novel inhibitor peptides so identified can be used in medical treatments and therapies directed against microbial infection. It is further contemplated that these novel inhibitor peptides can be used, in turn, to identify additional novel antibacterial peptides using a synthetic approach. The coding sequence of the inhibitory peptides is determined, and peptides are then chemically synthesized and tested in the host cell for their inhibitory properties.

Novel inhibitor peptides identified in a pathogenic microbial host cell according to the invention can also be used to elucidate potential new drug targets. The protein target that the inhibitor peptide inactivated is identified using reverse genetics by isolating mutants that are no longer inhibited by the peptide. These mutants are then mapped in order to precisely determine the protein target that is inhibited. New antibacterial drugs can then be developed using various known or yet to be discovered pharmaceutical strategies.

Following transformation of the host cell, the transformed host cell is initially grown under conditions that repress expression of the peptide. Expression of the peptide is then induced. For example, when a *lac* promoter operator system is used for expression, IPTG is added to the culture medium. A determination is subsequently made as to whether the peptide is inhibitory to host cell growth, wherein inhibition of host cell growth under induced but not repressed conditions is indicative of the expression of a bioactive peptide.

Notably, the bioactive peptides identified according to the method of the invention are, by reason of the method itself, stable in the intracellular environment of the host cell. The method of the invention thus preferably identifies bioactive peptides that are resistant to proteases and peptidases. Resistance to proteases and peptidases can be evaluated by measuring peptide degradation when in contact with appropriate cell extracts or purified peptidases and/or proteases, employing methods well-known in the art. A protease- or peptidase-resistant peptide is evidenced by a longer half-life in the presence of proteases or peptidases compared to a control peptide.

Randomized peptides used in the screening method of the invention can be optionally engineered according to the method of the invention in a biased synthesis to increase their stability by making one or both of the N-terminal or C-terminal ends more resistant to proteases and peptidases, and/or by engineering into the peptides a stabilizing motif.

In one embodiment of the screening method of the invention, the putative bioactive peptide is stabilized by adding a stabilizing group to the N-terminus, the C-terminus, or to both termini. To this end, the nucleic acid sequence that encodes the randomized peptide in the expression vector or the expression vector itself is preferably modified to encode a first stabilizing group comprising the N-terminus of the peptide, and a second stabilizing group comprising the C-terminus of the peptide.

The stabilizing group can be a stable protein, preferably a small stable protein such as thioredoxin, glutathione sulfotransferase, maltose binding protein, glutathione reductase, or a four-helix bundle protein such as Rop protein, although no specific size limitation on the protein anchor is intended.

Proteins suitable for use as a stabilizing group can be either naturally occurring or non-naturally occurring. They can be isolated from an endogenous source, chemically or enzymatically synthesized, or produced using recombinant DNA technology. Proteins that are particularly well-suited for use as a stabilizing group are those that are relatively short in length and form very stable structures in solution. Proteins having molecular weights of less than about 50 kD are preferred for use as a stabilizing group; more preferably the molecular weight of the small stable protein is less than about 25 kD, most preferably less than about 12 kD. For example, *E. coli* thioredoxin has a molecular weight about 11.7 kD; *E. coli* glutathione sulfotransferase has a molecular weight of about 22.9 kD, and Rop from the ColE1 replicon has a molecular weight of about 7.2 kD; and maltose binding protein (without its signal sequence) is about 40.7 kD. The small size of the Rop protein makes it especially useful as a stabilizing group, fusion partner, or peptide anchor, in that it is less likely than larger proteins to interfere with the accessibility of the linked peptide, thus preserving its bioactivity. Rop's highly ordered anti-parallel four-helix bundle topology (after dimerization) and slow unfolding kinetics (see, e.g., Betz et al. *Biochemistry* **36**, 2450-2458 (1997)) also contribute to its usefulness as a peptide anchor according to the invention. Other proteins with similar folding kinetics and/or thermodynamic stability (e.g., Rop has a midpoint temperature of denaturation, T_m , of about 71°C, Steif et al., *Biochemistry* **32**, 3867-3876 (1993)) are also preferred peptide anchors. Peptides or proteins having highly stable tertiary motifs, such as a four-helix bundle topology, are particularly preferred.

Alternatively, the stabilizing group can constitute one or more prolines (Pro). Preferably, a proline dipeptide (Pro-Pro) is used as a stabilizing group, however additional prolines may be included. The encoded proline(s) are typically naturally occurring amino acids, however if and to the extent a proline derivative, for example a hydroxyproline or a methyl- or ethyl- proline derivative, can be encoded or otherwise incorporated into the peptide, those proline derivatives are also useful as stabilizing groups.

At the N-terminus of the peptide, the stabilizing group can alternatively include an oligopeptide having the sequence Xaa-Pro_m-, wherein Xaa is any amino acid and m is greater than 0. Preferably, m is about 1 to about 5.

preferably $m = 2$ or 3 , more preferably, $m = 2$. Likewise, at the C-terminus of the peptide, the stabilizing group can alternatively include an oligopeptide having the sequence $-\text{Pro}_m\text{-Xaa}$, wherein Xaa is any amino acid, and m is greater than 0. Preferably, n is about 1 to about 5; preferably $n = 2$ or 3 , more

- 5 preferably, $m = 2$. In a particularly preferred embodiment of the method of the invention, the nucleic acid sequence that encodes the randomized peptide in the expression vector is modified to encode each of a first stabilizing group comprising the N-terminus of the peptide, the first stabilizing group being selected from the group consisting of small stable protein, Pro-, Pro-Pro-, Xaa-Pro- and Xaa-Pro-Pro-, and a second stabilizing group comprising the C-terminus of the peptide, the second stabilizing group being selected from the group consisting of a small stable protein, -Pro-, -Pro-Pro-, Pro-Xaa and Pro-Pro-Xaa. The resulting peptide has enhanced stability in the intracellular environment relative to a peptide lacking the terminal stabilizing groups.

- 15 In another preferred embodiment of the screening method of the invention, the expression vector encodes a four-helix bundle protein fused, at either the C-terminus or the N-terminus, to the randomized peptide. Preferably, the four-helix bundle protein is *E. coli* Rop protein or a homolog thereof. The non-fused terminus of the randomized peptide can, but need not, comprise a stabilizing group. The resulting fusion protein is predicted to be more stable than the randomized peptide itself in the host intracellular environment. Where the four-helix bundle protein is fused to the N-terminus, the randomized peptide can optionally be further stabilized by engineering one or more prolines, with or without a following undefined amino acid (e.g., -Pro-, -Pro-Pro-, -Pro-Xaa, -Pro-Pro-Xaa, etc.) at the C-terminus of the peptide sequence; likewise, when the four-helix bundle protein is fused to the C-terminus, the randomized peptide can be further stabilized by engineering one or more prolines, with or without a preceding undefined amino acid (e.g., Pro-, Pro-Pro-, Xaa-Pro-, Xaa-Pro-Pro-, etc.) at the N-terminus of the peptide sequence.

- 25 30 In yet another embodiment of the screening method of the invention, the putative bioactive peptide is stabilized by engineering into the peptide a stabilizing motif such as an α -helix motif or an opposite charge ending motif. Chemical synthesis of the oligonucleotide according to the scheme

[(CAG)A(TCAG)] yields an oligonucleotide encoding a peptide consisting of a random mixture of the hydrophilic amino acids His, Gln, Asn, Lys, Asp, and Glu (see Table 14). Except for Asp, these amino acids are most often associated with α -helical secondary structural motifs; the resulting oligonucleotides are thus biased in favor of oligonucleotides that encode peptides that are likely to form α -helices in solution. Alternatively, the putative bioactive peptide is stabilized by flanking a randomized region with a region of uniform charge (e.g., positive charge) on one end and a region of opposite charge (e.g., negative) on the other end, to form an opposite charge ending motif. To this end, the nucleic acid sequence that encodes the randomized peptide in the expression vector or the expression vector itself is preferably modified to encode a plurality of sequential uniformly charged amino acids comprising the N-terminus of the peptide, and a plurality of sequential oppositely charged amino acids comprising the C-terminus of the peptide. The positive charges are supplied by a plurality of positively charged amino acids consisting of lysine, histidine, arginine or a combination thereof; and the negative charges are supplied by a plurality of negatively charged amino acids consisting of aspartate, glutamate or a combination thereof. It is expected that such a peptide will be stabilized by the ionic interaction of the two oppositely charged ends. Preferably, the putative bioactive peptide contains at least three charged amino acids at each end. More preferably, it contains at least four charged amino acids at each end. In a particularly preferred embodiment, the larger acidic amino acid glutamate is paired with the smaller basic amino acid lysine, and the smaller acidic amino acid aspartate is paired with the larger basic amino acid arginine.

It is to be understood that novel bioactive peptides identified using the method for identification of bioactive peptides described herein are also included in the present invention.

The present invention further provides a bioactive peptide containing one or more structural features or motifs selected to enhance the stability of the bioactive peptide in an intracellular environment. During development and testing of the intracellular screening method of the present invention, it was surprisingly discovered that several bioactive peptides identified from the randomized peptide library shared particular structural features. For example, a disproportionately high number of bioactive peptides identified using the

intracellular screening method contained one or more proline residues at or near a peptide terminus. A disproportionate number also contained sequences predicted, using structure prediction algorithms well-known in the art, to form secondary structures such as α helices or β sheets; or a hydrophobic membrane spanning domain. Bioactive fusion proteins comprising the randomized peptide sequence fused to the Rop protein, due to a deletion event in the expression vector, were also identified.

Accordingly, the invention provides a bioactive peptide having a stabilizing group at its N-terminus, its C-terminus, or at both termini. In a bioactive peptide stabilized at only one terminus (i.e., at either the N- or the C-terminus) the stabilizing group is preferably either a four-helix bundle protein, such as Rop protein, a proline (Pro), or a proline dipeptide (Pro-Pro). It should be understood that in any synthetic peptide having a stabilizing group that includes one or more prolines according to the present invention, the proline is preferably a naturally occurring amino acid; alternatively, it can be a synthetic derivative of proline, for example a hydroxyproline or a methyl- or ethyl- proline derivative. Accordingly, where the abbreviation "Pro" is used herein in connection with a stabilizing group that is part of a synthetic peptide, it is meant to include proline derivatives in addition to a naturally occurring proline.

A peptide stabilized at both termini includes a first stabilizing group comprising the N-terminus, and a second stabilizing group stabilizing the C-terminus, where the first and second stabilizing groups are as defined previously in connection with the method for identifying bioactive peptides. The stabilizing group is covalently attached to the peptide. The bioactive peptide of the invention includes a bioactive peptide that has been detectably labeled, derivatized, or modified in any manner desired prior to use, provided it contains one or more terminal stabilizing groups as provided herein. In one preferred embodiment of the bioactive peptide of the invention, the first stabilizing group, comprising the N-terminus, is Xaa-Pro-Pro-, Xaa-Pro-, Pro- or Pro-Pro-; and second stabilizing group, comprising the C-terminus, is Pro-Pro-Xaa, -Pro-Xaa, -Pro or -Pro-Pro; preferably -Pro-Pro. In another preferred embodiment, the first (N-terminal) stabilizing group is a small stable protein, preferably a four-helix bundle protein such as Rop protein; and the second (C-terminal) stabilizing

group is Pro-Pro-Xaa, -Pro-Xaa, -Pro or -Pro-Pro; preferably -Pro-Pro. In yet another preferred embodiment, the second (C-terminal) stabilizing group is a small stable protein, preferably a four-helix bundle protein such as Rop protein, and the first (N-terminal) stabilizing group is Pro-, Pro-Pro-, Xaa-Pro- or Xaa-Pro-Pro-.

The invention further provides a peptide stabilized by flanking the amino acid sequence of a bioactive peptide with an opposite charge ending motif, as described herein. Preferably, the resulting stabilized peptide retains at least a portion of the biological activity of the bioactive protein. The stabilized peptide includes a peptide that has been detectably labeled, derivatized, or modified in any manner desired prior to use.

It should be understood that any bioactive peptide, without limitation, can be stabilized according to the invention by attaching a stabilizing group to either or both of the N- and C-termini, or by attaching oppositely charged groups to the N- and C-termini to form an opposite charge ending motif. Included in the present invention are any and various antimicrobial peptides, inhibitory peptides, therapeutic peptide drugs, and the like, as, for example and without limitation, those listed in Tables 1 and 2, that have been modified at one or both peptide termini to include a stabilizing group, for example a four-helix bundle protein such as Rop protein, proline (Pro-), a proline-proline dipeptide (Pro-Pro-), an Xaa-Pro- dipeptide, or an Xaa-Pro-Pro-tripeptide at the N-terminus, and/or a four-helix bundle protein such as Rop protein, proline (-Pro), or a proline-proline dipeptide (-Pro-Pro), a Pro-Xaa dipeptide, or a Pro-Pro-X tripeptide at the C-terminus; or that have been modified to contain an opposite charge ending motif according to the invention. In this aspect the invention is exemplified by peptides such as Pro-Pro-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Ile-Pro-Pro (SEQ ID NO: 3) and Glu-Asp-Glu-Asp-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Ile-Arg-Lys-Arg-Lys (SEQ ID NO: 4), wherein the middle nine amino acids (-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Ile-; SEQ ID NO: 5) constitute the sequence of angiotensin.

Modification of a bioactive peptide to yield a stabilized bioactive peptide according to the invention can be achieved by standard techniques well-known in the arts of genetics and peptide synthesis. For example, where the peptide is

synthesized *de novo*, as in solid state peptide synthesis, one or more prolines can be added at the beginning and the end of the peptide chain during the synthetic reaction. In recombinant synthesis, for example as described in Example III herein, one or more codons encoding proline can be inserted into the peptide coding sequence at the beginning and or the end of the sequence, as desired. Preferably, codons encoding N-terminal prolines are inserted after (i.e., 3' to) the initiation site ATG (which encodes for methionine). Analogous techniques are used to synthesize bioactive peptides having an opposite charge ending motif. When a known bioactive peptide is modified to yield a stabilized bioactive peptide according to the invention, the unmodified peptide can conveniently be used as a control in a protease- or peptidase-resistance assay as described hereinabove to confirm, if desired, that the modified peptide exhibits increased stability.

The present invention also provides a cleavable polypeptide comprising a stabilized, bioactive peptide either immediately preceded by (i.e., adjacent to the N-terminus of the bioactive peptide) a cleavage site, or immediately followed by (i.e., adjacent to the C-terminus of the bioactive peptide) a cleavage site. Thus, a bioactive peptide as contemplated by the invention can be part of a cleavable polypeptide. The cleavable polypeptide is cleavable, either chemically, as with cyanogen bromide, or enzymatically, to yield the bioactive peptide. The resulting bioactive peptide either includes a first stabilizing group comprising its N-terminus and a second stabilizing group comprising its C-terminus, or it includes an opposite charge ending motif, both as described hereinabove. The cleavage site immediately precedes the N-terminal stabilizing group or immediately follows the C-terminal stabilizing group. In the case of a bioactive peptide having an opposite charge ending motif, the cleavage site immediately precedes the first charged region or immediately follows the second charged region. The cleavage site makes it possible to administer a bioactive peptide in a form that could allow intracellular targeting and/or activation.

Alternatively, a bioactive peptide of the invention can be fused to a noncleavable N-terminal or C-terminal targeting sequence wherein the targeting sequence allows targeted delivery of the bioactive peptide, e.g., intracellular targeting or tissue-specific targeting of the bioactive peptide. In one

embodiment of this aspect of the invention, the free terminus of the bioactive peptide comprises a stabilizing group as described hereinabove in connection with the screening method for identifying bioactive peptides, for example one or more prolines. The targeting sequence forming the other peptide terminus can, but need not, contain a small stable protein such as Rop or one or more prolines comprising its terminus, as long as the targeting function of the targeting sequence is preserved. In another embodiment of this aspect of the invention, the bioactive peptide comprises a charge ending motif as described hereinabove, wherein one charged region occupies the free terminus of the bioactive peptide, and the other charged region is disposed between the targeting sequence and the active sequence of the bioactive peptide.

The invention further includes a method for using an antimicrobial peptide that includes covalently linking a stabilizing group, as described hereinabove, to the N-terminus, the C-terminus, or to both termini, to yield a stabilized antimicrobial peptide, then contacting a microbe with the stabilized antimicrobial peptide. Alternatively, the stabilized antimicrobial peptide used in this aspect of the invention is made by covalently linking oppositely charged regions, as described hereinabove, to each end of the antimicrobial peptide to form an opposite charge ending motif. An antimicrobial peptide is to be broadly understood as including any bioactive peptide that adversely affects a microbe such as a bacterium, virus, protozoan, or the like, as described in more detail hereinabove. An example of an antimicrobial peptide is an inhibitory peptide that inhibits the growth of a microbe. When the antimicrobial peptide is covalently linked to a stabilizing group at only one peptide terminus, any of the stabilizing groups described hereinabove can be utilized. When the antimicrobial peptide is covalently linked to a stabilizing group at both peptide termini, the method includes covalently linking a first stabilizing group to the N-terminus of the antimicrobial peptide and a second stabilizing group to the C-terminus of the antimicrobial peptide, where the first and second stabilizing groups are as defined previously in connection with the method for identifying bioactive peptides. In a preferred embodiment of the method for using an antimicrobial peptide, one or more prolines, more preferably a proline-proline dipeptide, is attached to at least one, preferably both, termini of the antimicrobial

peptide. Alternatively, or in addition, an Xaa-Pro- or an Xaa-Pro-Pro sequence can be attached to the N-terminus of a microbial peptide, and or a Pro-Xaa or a Pro-Pro-Xaa sequence can be attached to the C-terminus, to yield a stabilized antimicrobial peptide.

5 The antimicrobial peptide thus modified in accordance with the invention has enhanced stability in the intracellular environment relative to an unmodified antimicrobial peptide. As noted earlier, the unmodified peptide can conveniently be used as a control in a protease- or peptidase-resistance assay as described hereinabove to confirm, if desired, that the modified peptide exhibits increased
10 stability. Further, the antimicrobial activity of the antimicrobial peptide is preferably preserved or enhanced in the modified antimicrobial peptide: modifications that reduce or eliminate the antimicrobial activity of the antimicrobial peptide are easily detected and are to be avoided.

 The invention further provides a method for inhibiting the growth of a
15 microbe comprising contacting the microbe with a stabilized inhibitory peptide. In one embodiment of this aspect of the invention, the stabilized inhibitory peptide has a stabilizing group at the N-terminus, the C-terminus, or to both. Preferably, the inhibitory peptide has a first stabilizing group comprising the N-terminus of the inhibitory peptide, and a second stabilizing group comprising the
20 C-terminus of the inhibitory peptide; the first and second stabilizing groups are as defined previously in connection with the method for identifying bioactive peptides. In another embodiment of this aspect of the invention, the inhibitory peptide is stabilized by the addition of oppositely charged regions to each end to form an opposite charge ending motif, as described hereinabove.

25 Also included in the present invention is a method for treating a patient having a condition treatable with a peptide drug, comprising administering to the patient a peptide drug that has been stabilized as described herein. Peptide drugs for use in therapeutic treatments are well-known (see Table 1). However, they are often easily degraded in biological systems, which affects their efficacy. In
30 on embodiment of the present method, the patient is treated with a stabilized drug comprising the peptide drug of choice and a stabilizing group attached at either the N-terminus, the C-terminus of, or at both termini of the peptide drug. In another embodiment of the present method, the patient is treated with a

stabilized drug comprising the peptide drug of choice and stabilized by attachment of oppositely charged regions to both termini of the peptide drug. Because the peptide drug is thereby stabilized against proteolytic degradation, greater amounts of the drug should reach the intended target in the patient.

5 In embodiments of the method involving administration of a peptide drug that is covalently linked to a stabilizing group at only one peptide terminus, the stabilizing group is preferably a four-helix bundle protein such as Rop protein, provided that attachment of the four-helix bundle protein to the peptide terminus preserves a sufficient amount of efficacy for the drug. It is to be nonetheless
10 understood that group or groups used to stabilize the peptide drug are as defined hereinabove, without limitation. In embodiments involving administration of a peptide drug covalently linked to a stabilizing group at both peptide termini, the peptide drug includes a first stabilizing group comprising the N-terminus of the peptide drug and a second stabilizing group comprising the C-terminus of the
15 peptide drug. Thus, in another preferred embodiment of the treatment method of the invention, the stabilized peptide drug comprises one or more prolines, more preferably a proline-proline dipeptide, attached to one or both termini of the peptide drug. For example, the peptide drug can be stabilized by covalent attachment of a Rop protein at one terminus, and by a proline or proline
20 dipeptide at the other terminus; in another preferred embodiment, the peptide drug can be stabilized by proline dipeptides at each of the N-terminus and C-terminus. Alternatively, or in addition, the stabilized peptide drug used in the treatment method comprises an Xaa-Pro- or an Xaa-Pro-Pro- sequence at the N-terminus of the peptide drug, and/or a -Pro-Xaa or a -Pro-Pro-Xaa sequence at
25 the C-terminus. Optionally, prior to administering the stabilized peptide drug, the treatment method can include a step comprising covalently linking a stabilizing group to one or both termini of the peptide drug to yield the stabilized peptide drug.

 If desired, the unmodified peptide drug can conveniently be used as a
30 control in a protease- or peptidase-resistance assay as described hereinabove to confirm that the stabilized peptide drug exhibits increased stability. Further, the therapeutic efficacy of the peptide drug is preferably preserved or enhanced in

the stabilized peptide drug; modifications that reduce or eliminate the therapeutic efficacy of the peptide drug are easily detected and are to be avoided.

The present invention further includes a fusion protein comprising a four-helix bundle protein, preferably Rop protein, and a polypeptide. Preferably the polypeptide is bioactive; more preferably it is a bioactive peptide. The fusion protein of the invention can be used in any convenient expression vector known in the art for expression or overexpression of a peptide or protein of interest. Optionally, a cleavage site is present between four helix bundle protein and the polypeptide to allow cleavage, isolation and purification of the polypeptide. In one embodiment of the fusion protein, the four helix bundle protein is covalently linked at its C-terminus to the N-terminus of the polypeptide; in an alternative embodiment, the four helix bundle protein is covalently linked at its N-terminus to the C-terminus of the polypeptide. Fusion proteins of the invention, and expression vectors comprising nucleic acid sequences encoding fusion proteins wherein the nucleic acid sequences are operably linked to a regulatory control element such as a promoter, are useful for producing or overproducing any peptide or protein of interest.

EXAMPLES

20

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

25

Example 1**Construction and characterization of a highly regulable expression vector, pLAC11, and its multipurpose derivatives, pLAC22 and pLAC33**

5

A number of different expression vectors have been developed over the years to facilitate the production of proteins in *E. coli* and related bacteria. Most of the routinely employed expression vectors rely on *lac* control in order to overproduce a gene of choice. While these vectors allow for overexpression of the gene product of interest, they are leaky due to changes that have been introduced into the *lac* control region and gene expression can never be shut off under repressed conditions, as described in more detail below. Numerous researchers have noticed this problem with the more popular expression vectors pKK223-3 (G. Posfai et al., *Gene*, 50: 63-67 (1986); N. Scrutton et al., *Biochem J.*, 245: 875-880 (1987)), pKK233-2 (P. Beremand et al., *Arch Biochem Biophys.*, 256: 90-100 (1987); K. Ooki et al., *Biochimie*, 76: 398-403 (1994)), pTrec99A (S. Ghosh, *Protein Expr. Purif.*, 10: 100-106 (1997); J. Ranie et al., *Mol. Biochem. Parasitol.*, 61: 159-169 (1993)), as well as the pET series (M. Eren et al., *J. Biol. Chem.*, 264: 14874-14879 (1989); G. Godson, *Gene* 100: 59-64 (1991)).

The expression vector described in this example, pLAC11, was designed to be more regulable and thus more tightly repressible when grown under repressed conditions. This allows better regulation of cloned genes in order to conduct physiological experiments. pLAC11 can be used to conduct physiologically relevant studies in which the cloned gene is expressed at levels equal to that obtainable from the chromosomal copy of the gene in question. The expression vectors described here were designed utilizing the wild-type *lac* promoter/operator in order to accomplish this purpose and include all of the *lac* control region, without modification, that is contained between the start of the O3 auxiliary operator through the end of the O1 operator. As with all *lac* based vectors, the pLAC11 expression vector described herein can be turned on or off by the presence or absence of the gratuitous inducer IPTG. In experiments in which a bacterial cell contained both a null allele in the chromosome and a

second copy of the wild-type allele on pLAC11, cells grown under repressed conditions exhibited the null phenotype while cells grown under induced conditions exhibited the wild-type phenotype. Thus the pLAC11 vector truly allows for the gene of interest to be grown under either completely repressed or
5 fully induced conditions. Two multipurpose derivatives of pLAC11, pLAC22 and pLAC33, were also constructed to fulfill different experimental needs.

The vectors pLAC11, pLAC22 and pLAC33 were deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA, 20110-2209, USA, on February 16, 1999, and assigned ATCC deposit
10 numbers ATCC 207108, ATCC 207110 and ATCC 207109, respectively. It is nonetheless to be understood that the written description herein is considered sufficient to enable one skilled in the art to fully practice the present invention. Moreover, the deposited embodiment is intended as a single illustration of one aspect of the invention and is not to be construed as limiting the scope of the
15 claims in any way.

MATERIALS AND METHODS

Media. Minimal M9 media (6 g disodium phosphate, 3 g potassium phosphate, 1 g ammonium chloride, 0.5 g sodium chloride, distilled water to 1L; autoclave; add 1 mL magnesium sulfate (1M) and 0.1 mL calcium chloride (1M); a sugar added to a final concentration of 0.2%; vitamins and amino acids as required for non-prototrophic strains) and rich LB media (10 g tryptone, 5 g yeast extract, 10 g sodium chloride, distilled water to 1L; autoclave) were prepared as described by Miller (J. Miller, "Experiments in molecular genetics" Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1972). The antibiotics ampicillin, kanamycin, streptomycin, and tetracycline (Sigma Chemical Company, St. Louis, MO) were used in rich media at a final concentration of 100, 40, 200, and 20 ug/ml, respectively. When used in minimal media, tetracycline was added at a final concentration of 10 ug/ml. 5-bromo-4-chloro-3-indoyl β -D-galactopyranoside (Xgal) was added to media at a final concentration of 40 ug/ml and unless otherwise noted. IPTG was added to media at a final concentration of 1 mM.

Chemicals and Reagents. When amplified DNA was used to construct the plasmids that were generated in this study, the PCR reaction was carried out using native *Pfu* polymerase from Stratagene (Cat. No. 600135). Xgal and IPTG were purchased from Diagnostic Chemicals Limited.

Bacterial Strains and Plasmids. Bacterial strains and plasmids are listed in Table 4. To construct ALS225, ALS224 was mated with ALS216 and streptomycin resistant, blue recombinants were selected on a Rich LB plate that contained streptomycin, Xgal, and IPTG. To construct ALS226, ALS224 was mated with ALS217 and streptomycin resistant, kanamycin resistant recombinants were selected on a Rich LB plate that contained streptomycin and kanamycin. To construct ALS515, ALS514 was mated with ALS216 and streptomycin resistant, blue recombinants were selected on a Rich LB plate that contained streptomycin, Xgal, and IPTG. To construct ALS527, ALS524 was mated with ALS224 and streptomycin resistant, tetracycline resistant

recombinants were selected on a Rich LB plate that contained streptomycin and tetracycline. To construct ALS535, ALS533 was mated with ALS498 and tetracycline resistant recombinants were selected on a Minimal M9 Glucose plate that contained tetracycline, leucine and thiamine (B₁) (Sigma Chemical Company). To construct ALS533, a P1 lysate prepared from *E. coli* strain K5076 (H. Miller et al., Cell **20**: 711-719 (1980)) was used to transduce ALS224 and tetracycline resistant transductants were selected.

Table 4: Bacterial strains and plasmids used in Example I

E. coli Strains

Laboratory Name	Original Name	Genotype	Source
AL S216	SF9100	<i>araD139 Δ(lac)U169 thi flhB5301 deoC7</i>	E. Altman et al., J. Biol. Chem. 265 :18148-18153 (1990)
AL S217	SF9100.1	<i>ptsF25 rpsE / F' lacP^l Z⁺ Y⁺ A⁺</i>	S. Emr (Univ. of California, San Diego)
AL S221	BL21(DE3)	<i>araD139 Δ(lac)U169 thi flhB5301 deoC7</i> <i>ptsF25 rpsE / F' lacP^l Z⁺ Tn5 Y⁺ A⁺</i>	F. Studier et al., J. Mol. Biol. 189 : 113-130 (1986)
AL S224	MC1061	<i>ompT hsdR(b) (R-M-) gal dem</i>	M. Casadaban et al., J. Mol. Biol. 138 :179-207 (1980).
AL S225		<i>araD139 Δ(ara:ABOIC-leu)7679 Δ(lac)X74</i> <i>galU galK rpsL hsr- hsm+</i>	This example
AL S226		MC1061 / F' lacP ^l Z ⁺ Y ⁺ A ⁺	This example
AL S269	CSE27	MC1061 / F' lacP ^l Z ⁺ Tn5 Y ⁺ A ⁺	J. Miller, "Experiments in molecular genetics" Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1972).
AL S413	MG1655	<i>E. coli</i> wild-type F-λ-	M. Guyer et al., Cold Spring Harbor Symp. Quant. Biol. 45 :135-140 (1980).
AL S498	JM101	<i>supE thi Δ(lac-proAB) / F' traD36 proA⁺ B⁺</i>	C. Yanisch-Perron et al., Gene. 33 : 103-119 (1985)
AL S514	NM1554	<i>lacP^l Δ(lacZ)M15</i> MC1061 <i>recA13</i>	E. Raleigh et al., Nucl. Acids Res. 16 : 1563-1575 (1988).
AL S515		MC1061 <i>recA13 / F' lacP^l Z⁺ Y⁺ A⁺</i>	This example
AL S524	XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44</i> (Cat. No. 200268)	Stratagene

ALS527	<i>relA1 lac / F' pro:AB lacP_l Δ(lacZ)M15</i> Tn10	This example
ALS533	MC1061 / F' <i>pro:AB lacP_l Δ(lacZ)M15</i> Tn10	This example
ALS535	MC1061 <i>pro:AB::Tn10</i> MC1061 <i>pro:AB::Tn10 / F' lacP_l</i> <i>Δ(lacZ)M15 pro:A⁺B⁺</i>	This example
ALS598	CAG18615 zjb-3179::Tn10dKan lambda- <i>rph-1</i>	M. Singer et al., Microbiol. Rev. 53: 1-24 (1989).

Plasmids

<u>Plasmid</u>	<u>Relevant Characteristics</u>	<u>Source</u>
<u>Name</u>		
pBH20	wild-type <i>lac</i> promoter / operator, Amp ^R , Tet ^R , colE1 replicon	K. Itakura et al., Science. 198 :1056-1063 (1977)
pBR322	Amp ^R , Tet ^R , colE1 replicon	F. Bolivar et al., Gene. 2 :95-113 (1977)
pET-21(+)	T7 promoter / <i>lac</i> operator, lacI ^q , Amp ^R , colE1 replicon	Novagen (Cat No. 69770-1)
pGE226	wild-type <i>trc:l</i> gene, Amp ^R	J. Weisemann, et al., J. Bacteriol. 163 :748-755 (1985)
pKK223-3	<i>lac</i> promoter / operator, Amp ^R , colE1 replicon	J. Brosius et al., Proc. Natl. Acad. Sci. USA. 81 :6929-6933 (1984)
pK2233-2	<i>trc</i> promoter / operator, Amp ^R , colE1 replicon	E. Amann et al., Gene. 40 :183-190 (1985)

pLysE	T7 lysozyme, Cam ^R , P15A replicon	F. Studier, J. Mol. Biol. 219 :37-44 (1991)
pLysS	T7 lysozyme, Cam ^R , P15A replicon	F. Studier, J. Mol. Biol. 219 :37-44 (1991)
pMS421	wild-type <i>lac</i> promoter / operator, <i>lac</i> ^{PA} , Strep ^R , Spec ^R , SC101 replicon	D. Graña et al., Genetics. 120 :319-327 (1988)
pTer7	wild-type <i>lacZ</i> coding region, Amp ^R	R. Young (Texas A&M University)
pTrc99A	<i>trc</i> promoter / operator, <i>lac</i> ^{PA} , Amp ^R , colE1 replicon	E. Amann et al., Gene. 69 :301-315 (1988)
pUC8	<i>lac</i> promoter / operator, Amp ^R , colE1 replicon	J. Vieira et al., Gene. 19 : 259-268 (1982)
pXf60	wild-type TOL pWWO <i>xyE</i> gene, Amp ^R	J. Westpheling (Univ. of Georgia)

Construction of the pLAC11, pLAC22, and pLAC33 expression vectors. To construct pLAC11, primers #1 and #2 (see Table 5) were used to PCR amplify a 952 base pair (bp) fragment from the plasmid pBH20 which contains the wild-type *lac* operon. Primer #2 introduced two different base pair mutations into the seven base spacer region between the Shine Dalgarno site and the ATG start site of the *lacZ* which converted it from AACAGCT to AAGATCT thus placing a Bgl II site in between the Shine Dalgarno and the start codon of the *lacZ* gene. The resulting fragment was gel isolated, digested with Pst I and EcoR I, and then ligated into the 3614 bp fragment from the plasmid pBR322ΔAval which had been digested with the same two restriction enzymes. To construct pBR322ΔAval, pBR322 was digested with Aval, filled-in using Klenow, and then religated. To construct pLAC22, a 1291 bp Nco I, EcoR I fragment was gel isolated from pLAC21 and ligated to a 4361 bp Nco I, EcoR I fragment which was gel isolated from pBR322/NcoI. To construct pLAC21, primers #2 and #3 (see Table 5) were used to PCR amplify a 1310 bp fragment from the plasmid pMS421 which contains the wild-type *lac* operon as well as the *lacI*^q repressor. The resulting fragment was gel isolated, digested with EcoR I, and then ligated into pBR322 which had also been digested with EcoR I. To construct pBR322/Nco I, primers #4 and #5 (see Table 5) were used to PCR amplify a 788 bp fragment from the plasmid pBR322. The resulting fragment was gel isolated, digested with Pst I and EcoR I, and then ligated into the 3606 bp fragment from the plasmid pBR322 which had been digested with the same two restriction enzymes. The pBR322/Nco I vector also contains added Kpn I and Sma I sites in addition to the new Nco I site. To construct pLAC33, a 2778 bp fragment was gel isolated from pLAC12 which had been digested with BsaB I and Bsa I and ligated to a 960 bp fragment from pUC8 which had been digested with Afl III, filled-in with Klenow, and then digested with Bsa I. To construct pLAC12, a 1310 bp Pst I, BamH I fragment was gel isolated from pLAC11 and ligated to a 3232 bp Pst I, BamH I fragment which was gel isolated from pBR322.

Table 5. Primers employed to PCR amplify DNA fragments that were used in the construction of the various plasmids described in Example 1

5	pLAC11 and pLAC22
1	(for) GTT GCG ATT GGT GCA GCG AT (SEQ ID NO:6)
2	(rev) ATT <u>GAA TTC</u> ATA <u>AGA TCT</u> TTC CTG TGT GAA ATT GTT ATC
	CGC (SEQ ID NO:7)
3	(for) ATT <u>GAA TTC</u> ACC ATG GAC ACC ATT GAA TGG TGC AAA A
10	(SEQ ID NO:8)
	pBR322/Nco I
4	(for) GTT GTT GCG ATT GGT GCA G (SEQ ID NO:9)
5	(rev) TGT ATG <u>AAT TCC</u> CGG GTA <u>GCA TTT</u> TTS AAG ACG AAA GGG
15	CCT C (SEQ ID NO:10)
	Bgl II - lacZ - Hind III
6	(for) TAC TAT <u>AGA TCT</u> ATG ACC ATG ATT ACG GAT TCA CTG (SEQ
	ID NO:11)
20	7 (rev) TAC ATA <u>AAG CTT</u> GGC CTG CCC GST TAT TAT TAT TTT (SEQ
	ID NO:12)
	Pst I - lacZ - Hind III
8	(for) TAT CAT <u>CTG CAG</u> AGG AAA CAG CTA TGA CCA TGA TTA CGG
25	ATT CAC TG (SEQ ID NO:13)
9	(rev) TAC ATA CTC GAG CAG GAA <u>AGC TTG</u> GCG TGC CCG GTT ATT
	ATT ATT TT (SEQ ID NO:14)
	BamH 1 - lacZ - Hind III (also uses primer #9)
30	10 (for) TAT CAT <u>GGA TCC</u> AGG AAA CAG CTA TGA CCA TGA TTA CGG
	ATT CAC TG (SEQ ID NO:15)
	Bgl II - recA - Hind III
11	(for) TAC TAT <u>AGA TCT</u> ATG GGT ATC GAC GAA AAC AAA CAG (SEQ
35	ID NO:16)
12	(rev) ATA TAT <u>AAG CTT</u> TTA AAA ATC TTC GTT AGT TTC TGC TAG
	G (SEQ ID NO:17)
	BamH 1 - xylE - EcoR I
40	13 (for) TAC TAT <u>AGA TCT</u> ATG AAC AAA GGT GTA ATG CGA CC (SEQ
	ID NO:18)
14	(rev) ATT AGT <u>GAA TTC</u> GCA CAA TCT CTG CAA TAA CTC GT (SEQ
	ID NO:19)

In Table 5 the regions of the primers that are homologous to the DNA target template are indicated with a dotted underline, while the relevant restriction sites are indicated with a solid underline. All primers are listed in the 5' → 3' orientation.

5

Compilation of the DNA sequences for the pLAC11, pLAC22, and pLAC33 expression vectors. All of the DNA that is contained in the pLAC11, pLAC22, and, pLAC33 vectors has been sequenced.

The sequence for the pLAC11 vector which is 4547 bp can be compiled as follows: bp 1-15 are AGATCTTATGAATTC (SEQ ID NO:20) from primer #2 (Table 5); bp 16-1434 are bp 4-1422 from pBR322 (GenBank Accession # J01749); bp 1435-1442 are TCGGTCGG, caused by filling in the *Ava* I site in pBR322Δ*Ava*I; bp 1443-4375 is bp 1427-4359 from pBR322 (GenBank Accession # J01749); and bp 4376-4547 are bp 1106-1277 from the wild-type *E. coli lac* operon (GenBank Accession # J01636).

The sequence for the pLAC22 vector which is 5652 bp can be compiled as follows: bp 1-15 are AGATCTTATGAATTC (SEQ ID NO:21) from primer #2 (Table 5); bp 16-4370 are bp 4-4358 from pBR322 (GenBank Accession # J01749); bp 4371-4376 is CCATGG which is the *Nco* I site from pBR322/*Nco* I; and bp 4377-5652 are bp 2-1277 from the wild-type *E. coli lac* operon (GenBank Accession # J01636), except that bp #4391 of the pLAC22 sequence or bp#16 from the wild-type *E. coli lac* operon sequence has been changed from a "C" to a "T" to reflect the presence of the *lac*⁷⁹ mutation (J. Brosius et al., Proc. Natl. Acad. Sci. USA, **81**:6929-6933 (1984)).

The sequence for the pLAC33 vector which is 3742 bp can be compiled as follows: bp 1-15 is AGATCTTATGAATTC (SEQ ID NO:22) from primer #2 (Table 5); bp 16-1684 are bp 4-1672 from pBR322 (GenBank Accession # J01749); bp 1685-2638 are bp 786-1739 from pUC8 (GenBank Accession # L09132); bp 2639-3570 are bp 3428-4359 from pBR322 (GenBank Accession # J01749); and bp 3571-3742 are bp 1106-1277 from the wild-type *E. coli lac* operon (GenBank Accession # J01636). In the maps for these vectors the *ori* is identified as per Balbás (P. Balbás et al., Gene, **50**:3-40 (1986)) while the *lacPO* is indicated starting with the O3 primary operator and ending with the O1

operator as per Müller-Hill (B. Müller-Hill, The *lac* Operon: A Short History of a Genetic Paradigm, Walter de Gruyter, Berlin, Germany (1996)).

Construction of the pLAC11-, pLAC22-, pLAC33-, pKK223-3-, pKK233-2-, pTrec99A-, and pET-21(+)-*lacZ* constructs. To construct pLAC11-*lacZ*, pLAC22-*lacZ*, and pLAC33-*lacZ*, primers #6 and #7 (see Table 5) were used to PCR amplify a 3115 bp fragment from the plasmid pTer7 which contains the wild-type *lacZ* gene. The resulting fragment was gel isolated, digested with Bgl II and Hind III, and then ligated into the pLAC11, pLAC22, or pLAC33 vectors which had been digested with the same two restriction enzymes. To construct pKK223-3-*lacZ* and pKK233-2-*lacZ*, primers #8 and #9 (see Table 5) were used to PCR amplify a 3137 bp fragment from the plasmid pTer7. The resulting fragment was gel isolated, digested with Pst I and Hind III, and then ligated into the pKK223-3 or pKK233-2 vectors which had been digested with the same two restriction enzymes. To construct pTrec99A-*lacZ* and pET-21(-)-*lacZ*, primers #9 and #10 (see Table 5) were used to PCR amplify a 3137 bp fragment from the plasmid pTer7. The resulting fragment was gel isolated, digested with BamH I and Hind III, and then ligated into the pTrec99A or pET-21(+) vectors which had been digested with the same two restriction enzymes.

Construction of the pLAC11-*recA* and *xyIE* constructs. To construct pLAC11-*recA*, primers #11 and #12 (see Table 5) were used to PCR amplify a 1085 bp fragment from the plasmid pGE226 which contains the wild-type *recA* gene. The resulting fragment was gel isolated, digested with Bgl II and Hind III, and then ligated into the pLAC11 vector which had been digested with the same two restriction enzymes. To construct pLAC11-*xyIE*, primers #13 and #14 (see Table 5) were used to PCR amplify a 979 bp fragment from the plasmid pXE60 which contains the wild-type *Pseudomonas putida xyIE* gene isolated from the TOL pWWO plasmid. The resulting fragment was gel isolated, digested with Bgl II and EcoR I, and then ligated into the pLAC11 vector which had been digested with the same two restriction enzymes.

Assays. β -galactosidase assays were performed as described by Miller (J. Miller, "Experiments in molecular genetics" Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1972)), while catechol 2,3-dioxygenase (catO₂ase) assays were performed as described by Zukowski, et. al. (M. Zukowski et al.,
5 Proc. Natl. Acad. Sci. U.S.A. **80**:1101-1105 (1983)).

RESULTS

Construction and features of pLAC11, pLAC22, and pLAC33. Plasmid
10 maps that indicate the unique restriction sites, drug resistances, origin of replication, and other relevant regions that are contained in pLAC11, pLAC22, and pLAC33 are shown in Figs. 2, 3 and 4, respectively. pLAC11 was designed to be the most tightly regulable of these vectors. It utilizes the ColE1 origin of replication from pBR322 and LacI repressor is provided in trans from either an
15 episome or another compatible plasmid. pLAC22 is very similar to pLAC11, however, it also contains *lacI^q*, thus a source of LacI does not have to be provided in trans. pLAC33 is a derivative of pLAC11 which utilizes the mutated ColE1 origin of replication from pUC8 (S. Lin-Chao et al., Mol. Micro. **6**:3385-3393 (1992)) and thus pLAC33's copy number is significantly higher than
20 pLAC11 and is comparable to that of other pUC vectors. Because the cloning regions of these three vectors are identical, cloned genes can be trivially shuffled between and among them depending on the expression demands of the experiment in question.

To clone into pLAC11, pLAC22, or pLAC33, PCR amplification is
25 performed with primers that are designed to introduce unique restriction sites just upstream and downstream of the gene of interest. Usually a Bgl II site is introduced immediately in front of the ATG start codon and an EcoR I site is introduced immediately following the stop codon. An additional 6 bases is added to both ends of the oligonucleotide in order to ensure that complete digestion of
30 the amplified PCR product will occur. After amplification the double-stranded (ds) DNA is restricted with Bgl II and EcoR I, and cloned into the vector which has also been restricted with the same two enzymes. If the gene of interest contains a Bcl I site, then Pst I and H I or Bcl I can be used instead since they

generate overhangs which are compatible with Bgl II. If the gene of interest contains an EcoR I site, then a site downstream of EcoR I in the vector (such as Hind III) can be substituted.

- 5 **Comparison of pLAC11, pLAC22, and pLAC33, to other expression vectors.** In order to demonstrate how regulable the pLAC11, pLAC22, and pLAC33 expression vectors were, the wild-type *lacZ* gene was cloned into pLAC11, pLAC22, pLAC33, pKK223-3, pKK233-2, pTrec99A, and pET-21(+). Constructs which required an extraneous source of LacI for their repression were
- 10 transformed into ALS225, while constructs which contained a source of LacI on the vector were transformed into ALS224. pET-21(+) constructs were transformed into BL21 because they require T7 RNA polymerase for their expression. Four clones were chosen for each of these seven constructs and β -galactosidase assays were performed under repressed and induced conditions.
- 15 Rich Amp overnights were diluted 1 to 200 in either Rich Amp Glucose or Rich Amp IPTG media and grown until they reached mid-log ($OD_{550} = 0.5$). In the case of pET-21(+) the pLysE and pLysS plasmids, which make T7 lysozyme and thus lower the amount of available T7 polymerase, were also transformed into each of the constructs. Table 6 shows the results of these studies and also lists
- 20 the induction ratio that was determined for each of the expression vectors. As the data clearly indicates, pLAC11 is the most regulable of these expression vectors and its induction ratio is close to that which can be achieved with the wild-type *lac* operon. The vector which yielded the lowest level of expression under repressed conditions was pLAC11, while the vector which yielded the
- 25 highest level of expression under induced conditions was pLAC33.

Table 6: β -galactosidase levels obtained in different expression vectors grown under either repressed or induced conditions

Vector	Source	# of Miller Units Observed		
		Repressed Conditions	Induced Conditions	Fold Induction
pLAC11	F'	19	11209	590X
pLAC22	Plasmid	152	13315	88X
pLAC33	F'	322	23443	73X
pKK223-3	F'	92	11037	120X
pKK233-2	F'	85	10371	122X
pTrec99A	Plasmid	261	21381	82X
pET-21(+)	Plasmid	2929	16803	6X
pET-21(+)/pLysE	Plasmid	4085	19558	5X
pET-21(+)/pLysS	Plasmid	1598	20268	13X

- 5 The average values obtained for the four clones that were tested from each vector are listed in the table. Standard deviation is not shown but was less than 5% in each case. Induction ratios are expressed as the ratio of enzymatic activity observed at fully induced conditions versus fully repressed conditions. The plasmid pLysE yielded unexpected results: it was expected to cause lower
- 10 amounts of lacZ to be expressed from pET-21(+) under repressed conditions and, instead, higher amounts were observed. As a result, both pLysE and pLysS were restriction mapped to make sure that they were correct.

- Demonstrating that pLAC11 constructs can be tightly regulated.** pLAC11
- 15 was designed to provide researchers with an expression vector that could be utilized to conduct physiological experiments in which a cloned gene is studied under completely repressed conditions where it is off or partially induced conditions where it is expressed at physiologically relevant levels. Figure 5 demonstrates how a pLAC11-lacZ construct can be utilized to mimic
- 20 chromosomally expressed lacZ that occurs under various physiological conditions by varying the amount of IPTG inducer that is added. ALS226 cells containing pLAC11-lacZ were grown to mid-log in rich media that contained

varying amounts of IPTG and then β -galactosidase activity was assayed. Also indicated in the graph are the average β -galactosidase activities obtained for strains with a single chromosomal copy of the wild-type *lacZ* gene that were grown under different conditions.

- 5 To demonstrate just how regulable pLAC11 is, the *recA* gene was cloned into the pLAC11 vector and transformed into cells which contained a null *recA* allele in the chromosome. As the results in Table 7 clearly shows, recombination can not occur in a host strain which contains a nonfunctional RecA protein and thus P1 lysates which provide a Tn10dKan transposon can not
- 10 be used to transduce the strain to Kan^R at a high frequency. *recA*⁻ cells which also contain the pLAC11-*recA* construct can be transduced to Kan^R at a high frequency when grown under induced conditions but can not be transduced to Kan^R when grown under repressed conditions.

15 **Table 7: The recombination (-) phenotype of a *recA* null mutant strain can be preserved with a pLAC11-*recA* (wild-type) construct under repressed conditions**

Strain	Repressed Conditions	Induced Conditions
	Number of Kan ^R transductants	Number of Kan ^R transductants
ALS225 (<i>recA</i> ⁺)	178,000	182,000
ALS514 (<i>recA</i> ⁻)	5	4
ALS515 (<i>recA</i> ⁻ pCyt-3- <i>recA</i>)	4	174,000

- 20 The data presented in Table 7 are the number of Kan^R transductants that were obtained from the different MC1061 derivative strains when they were transduced with a P1 lysate prepared from strain ALS598 which harbored a Tn10dKan transposon insertion. Overnights were prepared from each of these strains using either rich medium to which glucose was added at a final
- 25 concentration of 0.2% (repressed conditions) or rich medium to which IPTG was added at a final concentration of 1 mM (induced conditions). The overnights

- were then diluted 1 to 10 into the same medium which contained CaCl_2 added to a final concentration of 10 mM and aerated for two hours to make them competent for transduction with P1 phage. Cells were then spectrophotometrically normalized and aliquots of 5 OD₅₅₀ cell equivalents in a volume of approximately 0.1 ml were transduced with 0.1 ml of concentrated P1 lysate as well as 0.1 ml of P1 lysates that had been diluted to 10^{-1} , 10^{-2} , or 10^{-3} . 0.2 ml of 0.1M Sodium Citrate was added to the cell phage mixtures and 0.2 ml of the final mixtures were plated onto Rich Kanamycin plates and incubated overnight at 37°C. The total number of Kan^R colonies were then counted.
- ALS225 *recA*⁺ data points were taken from the transductions which used the 10^{-3} diluted phage, while ALS514 *recA*⁻ data points were taken from the transductions which used the concentrated phage. The data points for ALS515 *recA*⁻ pCyt-3-*recA* grown under repressed conditions were taken from the transductions which used the concentrated phage, while the data points for ALS515 *recA*⁻ pCyt-3-*recA* grown under induced conditions were taken from the transductions which used the 10^{-3} diluted phage.

Testing various sources of LacI for trans repression of pLAC11. Because pLAC11 was designed to be used with an extraneous source of LacI repressor, different episomal or plasmid sources of LacI which are routinely employed by researchers were tested. Since one of the LacI sources also contained the *lacZ* gene, a reporter construct other than pLAC11-*lacZ* was required and thus a pLAC11-*xyIE* construct was engineered. Table 8 shows the results of this study.

All of the LacI sources that were tested proved to be adequate to repress expression from pLAC11, however, some were better than others. The basal level of expression that was observed with F's which provided *lacI*^{q1} or with the plasmid pMS421 which provided *lacI*^q at approximately six copies per cell was lower than the basal level of expression that was observed with F's which provided *lacI*^q all three times that the assay was run. Unfortunately, however, the *xyIE* gene could not be induced as high when *lacI*^{q1} on a F' or *lacI*^q on a plasmid was used as the source of Lac repressor.

Table 8: Catechol 2,3-dioxygenase levels obtained for a pLAC11-*xylE* construct when Lac repressor is provided by various sources

5

Strain	Source of LacI	Catechol 2,3-dioxygenase activity in milliunits/mg	
		Repressed Conditions	Induced Conditions
ALS224	None	32.7	432.8
ALS535	F' <i>lacI</i> ^q Δ (<i>lacZ</i>)M15 <i>proA</i> + <i>B</i> + Tn10	.3	204.4
ALS527	F' <i>lacI</i> ^q Δ (<i>lacZ</i>)M15 <i>proA</i> + <i>B</i> +	.3	243.3
ALS227	pMS421 <i>lacI</i> ^q	.2	90.9
ALS225	F' <i>lacI</i> ^q 1 <i>Z</i> ⁺ <i>Y</i> ⁺ <i>A</i> ⁺	.2	107.4
ALS226	F' <i>lacI</i> ^q 1 <i>Z</i> ::Tn5 <i>Y</i> ⁺ <i>A</i> ⁺	.2	85.1

The wild-type *xylE* gene was cloned into the pLAC11 vector and the resulting pLAC11-*xylE* construct was then transformed into each of the MC1061 derivative strains listed in the table. Rich overnights were diluted 1 to 200 in
 10 either Rich Glucose or Rich IPTG media and grown until they reached mid-log (OD₅₅₀ = 0.5). Cell extracts were then prepared and catechol 2,3-dioxygenase assays were performed as described by Zukowski, et. al (Proc. Natl. Acad. Sci. U.S.A. 80:1101-1105 (1983)). The average values obtained in three different experiments are listed in the table. Standard deviation is not shown but was less
 15 than 10% in each case.

DISCUSSION

Most of the routinely employed expression vectors rely on *lac* control in
 20 order to overproduce a gene of choice. The *lac* promoter/operator functions as it does due to the interplay of three main components. First, the wild-type *lac* -10

region (TATGTT) is very weak. c-AMP activated CAP protein is able to bind to the CAP site just upstream of the -35 region which stimulates binding of RNA polymerase to the weak -10 site. Repression of the *lac* promoter is observed when glucose is the main carbon source because very little c-AMP is present
5 which results in low amounts of available c-AMP activated CAP protein. When poor carbon sources such as lactose or glycerol are used, c-AMP levels rise and large amounts of c-AMP activated CAP protein become available. Thus induction of the *lac* promoter can occur. Second, Lac repressor binds to the *lac* operator. Lac repressor can be overcome by allolactose which is a natural
10 byproduct of lactose utilization in the cell, or by the gratuitous inducer, IPTG. Third, the *lac* operator can form stable loop structures which prevents the initiation of transcription due to the interaction of the Lac repressor with the *lac* operator (O1) and one of two auxiliary operators, O2 which is located downstream in the coding region of the *lacZ* gene, or O3 which is located just
15 upstream of the CAP binding site.

While binding of Lac repressor to the *lac* operator is the major effector of *lac* regulation, the other two components are not dispensable. However, most of the routinely used *lac* regulable vectors either contain mutations or deletions which alter the affect of the other two components. The pKK223-3 (J. Brosius et
20 al., Proc. Natl. Acad. Sci. USA. **81**:6929-6933 (1984)), pKK233-2 (E. Amann et al., Gene. **40**:183-190 (1985)), pTrc99A (E. Amann et al., Gene. **69**:301-315 (1988)), and pET family of vectors (F. Studier, Method Enzymol. **185**:60-89 (1990)) contain only the *lac* operator (O1) and lack both the CAP binding site as well as the O3 auxiliary operator. pKK223-3, pKK233-2, and pTrc99 use a *trp-lac*
25 *lac* hybrid promoter that contains the *trp* -35 region and the *lacUV5* -10 region which contains a strong TATAAT site instead of the weak TATGTT site. The pET family of vectors use the strong T7 promoter. Given this information, perhaps it is not so surprising researchers have found it is not possible to tightly shut off genes that are cloned into these vectors.

30 The purpose of the studies described in Example I was to design a vector which would allow researchers to better regulate their cloned genes in order to conduct physiological experiments. The expression vectors described herein were designed utilizing the wild-type *lac* promoter/operator in order to

accomplish this purpose and include all of the *lac* control region, without modification, that is contained between the start of the O3 auxiliary operator through the end of the O1 operator. As with all *lac* based vectors, the pLAC11, pLAC22, and pLAC33 expression vectors can be turned on or off by the
5 presence or absence of the gratuitous inducer IPTG.

Because the new vector, pLAC11, relies on the wild-type *lac* control region from the auxiliary *lac* O3 operator through the *lac* O1 operator, it can be more tightly regulated than the other available expression vectors. In direct comparison studies with pKK223-3, pKK233-2, pTrc99A, and pET-21(+), the
10 lowest level of expression under repressed conditions was achievable with the pLAC11 expression vector. Under fully induced conditions, pLAC11 expressed *lacZ* protein that was comparable to the levels achievable with the other expression vectors. Induction ratios of 1000x have been observed with the wild-type *lac* operon. Of all the expression vectors that were tested, only pLAC11
15 yielded induction ratios which were comparable to what has been observed with the wild-type *lac* operon. It should be noted that the regulation achievable by pLAC11 may be even better than the data in Table 6 indicates. Because *lacZ* was used in this test, the auxiliary *lac* O2 operator which resides in the coding region of the *lacZ* gene was provided to the pKK223-3, pKK233-2, pTrc99A,
20 and pET-21(+) vectors which do not normally contain either the O2 or O3 auxiliary operators. Thus the repressed states that were observed in the study in Table 6 are probably lower than one would normally observe with the pKK223-3, pKK233-2, pTrc99A, and pET-21(+) vectors.

To meet the expression needs required under different experimental
25 circumstances, two additional expression vectors which are derivatives of pLAC11 were designed. pLAC22 provides *lacI* on the vector and thus unlike pLAC11 does not require an extraneous source of LacI for its repression. pLAC33 contains the mutated ColE1 replicon from pUC8 and thus allows proteins to be expressed at much higher levels due to the increase in the copy
30 number of the vector. Of all the expressions that were evaluated in direct comparison studies, the highest level of protein expression under fully induced conditions was achieved using the pLAC33 vector. Because the cloning regions are identical in pLAC11, pLAC22, and pLAC33, genes that are cloned into one

of these vectors can be trivially subeloned into either of the other two vectors depending on experimental circumstances. For physiological studies, pLAC11 is the best suited of the three vectors. If, however, the bacterial strain of choice can not be modified to introduce elevated levels of Lac repressor protein which
5 can be achieved by F's or compatible plasmids that provide *lacI^q* or *lacI^{q1}*, the pLAC22 vector can be utilized. If maximal overexpression of a gene product is the goal, then the pLAC33 vector can be utilized.

Numerous experiments call for expression of a cloned gene product at physiological levels; i.e., at expression levels that are equivalent to the
10 expression levels observed for the chromosomal copy of the gene. While this is not easily achievable with any of the commonly utilized expression vectors, these kinds of experiments can be done with the pLAC11 expression vector. By varying the IPTG concentrations, expression from the pLAC11 vector can be adjusted to match the expression levels that occur under different physiological
15 conditions for the chromosomal copy of the gene. In fact, strains which contain both a chromosomal null mutation of the gene in question and a pLAC11 construct of the gene preserve the physiological phenotype of the null mutation under repressed conditions.

Because the use of Lac repressor is an essential component of any
20 expression vector that utilizes the *lac* operon for its regulation, the ability of different source of LacI to repress the pLAC11 vector was also investigated. Researchers have historically utilized either *lacI^q* constructs which make 10 fold more Lac repressor than wild-type *lacI* or *lacI^{q1}* constructs which make 100 fold more Lac repressor than wild-type *lacI* (B. Müller-Hill, Prog. Biophys. Mol.
25 Biol. **30**:227-252 (1975)). The greatest level of repression of pLAC11 constructs could be achieved using F's which provided approximately one copy of the *lacI^{q1}* gene or a multicopy compatible plasmid which provided approximately six copies of the *lacI^q* gene. However, the induction that was achievable using these *lacI* sources was significantly lower than what could be achieved when F's
30 which provided approximately one copy of the *lacI^q* gene were used to repress the pLAC11 construct. Thus if physiological studies are the goal of an investigation, then F's which provide approximately one copy of the *lacI^{q1}* gene

or a multicopy compatible plasmid which provides approximately six copies of the *lacI^q* gene can be used to regulate the pLAC11 vector. However, if maximal expression is desired, then F's which provide approximately one copy of the *lacI^q* gene can be utilized. Alternatively, if a bacterial strain can tolerate
5 prolonged overexpression of an expressed gene and overexpression of a gene product is the desired goal, then maximal expression under induced conditions is obtained when a bacteria strain lacks any source of Lac repressor.

Example II

10

An *in vivo* approach for generating novel bioactive peptides that inhibit the growth of *E. coli*

A randomized oligonucleotide library containing sequences capable of
15 encoding peptides containing up to 20 amino acids was cloned into pLAC11 (Example I) which allowed the peptides to either be tightly turned off or overproduced in the cytoplasm of *E. coli*. The randomized library was prepared using a [NNN] codon design instead of either the [NN(G,T)] or [NN(G,C)] codon design used by most fusion-phage technology researchers. [NN(G,T)] or
20 [NN(G,C)] codons have been widely used instead of [NNN] codons to eliminate two out of the three stop codons, thus increasing the amount of full-length peptides that can be synthesized without a stop codon (J. Scott et al., Science **249**:386-390 (1990); J. Delvin et al., Science **249**:404-406 (1990); S. Cwirla et al., Proc. Nat'l. Acad. Sci. U.S.A. **87**:6378-6382 (1990)). However, the
25 [NN(G,T)] and [NN(G,C)] oligonucleotide codon schemes eliminate half of the otherwise available codons and, as a direct result, biases the distribution of amino acids that are generated. Moreover, the [NN(G,T)] and [NN(G,C)] codon schemes drastically affect the preferential codon usage of highly expressed genes and removes a number of the codons which are utilized by the abundant tRNAs
30 that are present in *E. coli* (H. Grosjean et al., Gene. **18**: 199-209 (1982); T. Ikemura, J. Mol. Biol. **151**: 389-409 (1981)).

Of the 20,000 peptides screened in this Example, 21 inhibitors of cell growth were found which could prevent the growth of *E. coli* on minimal media.

The top twenty inhibitor peptides were evaluated for strength of inhibition, and the putative amino acid sequences of the top 10 "anchorless" inhibitor peptides were examined for commonly shared features or motifs.

5 MATERIALS AND METHODS

Media. Rich LB and minimal M9 media used in this study was prepared as in Example I. Ampicillin was used in rich media at a final concentration of 100 ug/ml and in minimal media at a final concentration of 50 ug/ml. IPTG was
10 added to media at a final concentration of 1 mM.

Chemicals and Reagents. Extension reactions were carried out using Klenow from New England Biolabs while ligation reactions were performed using T4 DNA Ligase from Life Sciences. IPTG was obtained from Diagnostic
15 Chemicals Limited.

Bacterial Strains and Plasmids. ALS225, which is MC1061/F'*lacI*^qZ⁺Y⁺A⁺ (see Example I), was the *E. coli* bacterial strain used in this Example. The genotype for MC1061 is *araD139* Δ (*araABOIC-leu*)7679 Δ (*lac*)X74 *galU galK*
20 *rpsL hsr- hsm+* (M. Casadaban et al., J. Mol. Biol. 138:179-207 (1980)). pLAC11, a highly regulable expression vector, is described in Example I.

Generation of the Randomized Peptide Library. The 93 base oligonucleotide 5' TAC TAT AGA TCT ATG (NNN)₂₀ TAA TAA GAA TTC TCG ACA 3'
25 (SEQ ID NO: 23), where N denotes an equimolar mixture of the nucleotides A, C, G, or T, was synthesized with the trityl group and subsequently purified with an OPC cartridge using standard procedures. The complementary strand of the 93 base oligonucleotide was generated by an extension/fill-in reaction with Klenow using an equimolar amount of the 18 base oligonucleotide primer 5'
30 TGT CGA GAA TTC TTA TTA 3' (SEQ ID NO: 24). After extension, the resulting ds-DNA was purified using a Promega DNA clean-up kit and restricted with EcoR 1 and Bgl II (Promega, Madison, WI). The digested DNA was again purified using a Promega DNA clean-up kit and ligated to pLAC11 vector which

had been digested with the same two restriction enzymes. The resulting library was transformed into electrocompetent ALS225 *E. coli* cells under repressed conditions (LB, ampicillin, plus glucose added to 0.2%).

Screening of Transformants to Identify Inhibitor Clones. Transformants were screened to identify any that could not grow on minimal media when the peptides were overproduced. Using this scheme, any transformant bacterial colony that overproduces a peptide that inhibits the production or function of a protein necessary for growth of that transformant on minimal media will be identified. Screening on minimal media, which imposes more stringent growth demands on the cell, will facilitate the isolation of potential inhibitors from the library. It is well known that growth in minimal media puts more demands on a bacterial cell than growth in rich media as evidenced by the drastically reduced growth rate; thus a peptide that adversely affects cell growth is more likely to be detected on minimal media. Screening was carried out using a grid-patching technique. Fifty clones at a time were patched onto both a rich repressing plate (LB Amp glucose) and a minimal inducing plate (M9 glycerol Amp IPTG) using an ordered grid. Patches that do not grow are sought because presumably these represent bacteria that are being inhibited by the expressed bioactive peptide. To verify that all of the inhibitors were legitimate, plasmid DNA was made from each inhibitory clone (QIA Prep Spin Miniprep kit; Qiagen Cat. No. 27104) and transformed into a fresh background (ALS225 cells), then checked to confirm that they were still inhibitory on plates and that their inhibition was dependent on the presence of the inducer, IPTG.

Growth Rate Analysis in Liquid Media. Inhibition strength of the peptides was assessed by subjecting the inhibitory clones to a growth rate analysis in liquid media. To determine the growth rate inhibition, starting cultures of both the peptides to be tested and a control strain which contains pLAC11 were diluted from a saturated overnight culture to an initial OD₅₅₀ of ~.01. All cultures were then induced with 1 mM IPTG and OD₅₅₀ readings were taken until the control culture reached an OD₅₅₀ of ~0.5. The hypothetical data in Table 9 shows that when the control strain reaches an OD₅₅₀ of about 0.54 (at

about 15 hours), a strain which contains a peptide that inhibits the growth rate at 50% will only have reached an OD₅₅₀ of only about 0.08. Thus, the growth of a 50% inhibited culture at 15 hours (i.e., the OD₅₅₀ at 15 hours, which is proportional to the number of cells in a given volume of culture) is only about 12.5% (that is, $0.08/0.64 \times 100$) of that of a control strain after the same amount of time, and the inhibitor peptide would thus have effectively inhibited the growth of the culture (as measured by the OD₅₅₀ at the endpoint) by 87.5% (=100% - 12.5%).

10 **Table 9: Hypothetical data from a peptide that inhibits growth rate at 30%, 50% and 70%**

Time in hours	OD550 readings on a control culture which contains pLAC11	OD550 readings on a culture which contains a peptide that inhibits the growth rate at...		
		25%	50%	75%
0	.010	.010	.010	.010
2.5	.020	.017	.015	.012
5	.040	.028	.020	.014
7.5	.080	.047	.030	.017
10	.160	.079	.040	.020
12.5	.320	.133	.060	.024
15	.640	.226	.080	.028

15 An example is shown in Fig. 6, wherein ALS225 cells containing the pLAC11 vector (control), and either the one day inhibitor pPep1 or the two day inhibitor pPep12 (see below), were grown in minimal M9 glycerol media with IPTG added to 1 mM. OD₅₅₀ readings were then taken hourly until the cultures had passed log phase. Growth rates were determined by measuring the 20 spectrophotometric change in OD₅₅₀ per unit time within the log phase of growth. The inhibition of the growth rate was then calculated for the inhibitors using pLAC11 as a control.

Sequencing the Coding Regions of the Inhibitor Peptide Clones. The 25 forward primer 5' TCA TTA ATG CAG CTG GCA CG 3' (SEQ ID NO: 25) and the reverse primer 5' TTC ATA CAC GGT GCC TGA CT 3' (SEQ ID NO: 26)

were used to sequence both strands of the top ten "anchorless" inhibitor peptide clones identified by the grid-patching technique. If an error-free consensus sequence could not be deduced from these two sequencing runs, both strands of the inhibitor peptide clones in question were resequenced using the forward
5 primer 5' TAG CTC ACT CAT TAG GCA CC 3' (SEQ ID NO: 27) and the reverse primer 5' GAT GAC GAT GAG CGC ATT GT 3' (SEQ ID NO: 28). The second set of primers were designed to anneal downstream of the first set of primers in the pLAC11 vector.

- 10 **Generating Antisense Derivatives of the Top Five "Anchorless" Inhibitor Clones.** Oligonucleotides were synthesized which duplicated the DNA insert contained between the Bgl II and EcoR I restriction sites for the top five "anchorless" inhibitor peptides as shown in Table 12 with one major nucleotide change. The "T" of the ATG start codon was changed to a "C" which resulted in
15 a ACG which can not be used as a start codon. The oligonucleotides were extended using the same 18 base oligonucleotide primer that was used to build the original peptide library. The resulting ds-DNA was then restricted, and cloned into pLAC11 exactly as described in the preceding section "Generating the randomized peptide library". The antisense oligonucleotides that were used
20 are as follows:

pPep1(antisense): 5' TAC TAT AGA TCT ACG GTC ACT GAA TTT TGT
GGC TTG TTG GAC CAA CTG CCT TAG TAA TAG TGG AAG GCT GAA
ATT AAT AAG AAT TCT CGA CA 3' (SEQ ID NO: 29);

25

pPep5(antisense): 5' TAC TAT AGA TCT ACG TGG CGG GAC TCA TGG
ATT AAG GGT AGG GAC GTG GGG TTT ATG GGT TAA AAT AGT TTG
ATA ATA AGA ATT CTC GAC A 3' (SEQ ID NO: 30)

- 30 pPep12(antisense): 5' TAC TAT AGA TCT ACG AAC GGC CGA ACC AAA
CGA ATC CGG GAC CCA CCA GCC GCC TAA ACA GCT ACC AGC TGT
GGT AAT AAG AAT TCT CGA CA 3' (SEQ ID NO: 31)

pPep13(antisense): 5' TAC TAT AGA TCT ACG GAC CGT GAA GTG ATG
TGT GCG GCA AAA CAG GAA TGG AAG GAA CGA ACG CCA TAG
GCC GCG TAA TAA GAA TTC TCG ACA 3' (SEQ ID NO: 32)

- 5 pPep19(antisense): 5' TAC TAT AGA TCT ACG AGG GGC GCC AAC TAA
GGG GGG GGG AAG GTA TTT GTC CCG TGC ATA ATC TCG GGT GTT
GTC TAA TAA GAA TTC TCG ACA 3' (SEQ ID NO: 33)

RESULTS

10

Identifying and Characterizing Inhibitor Peptides from the Library.

Approximately 20,000 potential candidates were screened as described hereinabove, and 21 IPTG-dependent growth inhibitors were isolated. All the inhibitors so identified were able to prevent the growth of the *E. coli* bacteria at
15 24 hours, and three of the 21 inhibitors were able to prevent the growth of the *E. coli* bacteria at 48 hours, using the grid patching technique. These three inhibitors were classified as "two day" inhibitors; the other 18 were classified as "one-day" inhibitors.

Results from the growth rate analysis for candidate peptide inhibitors are
20 shown in Table 10. The % inhibition of the growth rate was calculated by comparing the growth rates of cells that contained induced peptides with the growth rate of cells that contained the induced pLAC11 vector. Averaged values of three independent determinations are shown.

Table 10: Ability of the Inhibitor Peptides to Inhibit Cell Growth

Inhibitor	Type	% Inhibition	Inhibitor	Type	% Inhibition
pLAC11 (control)	---	0	Ppep11	1 Day	22
pPep1	1 Day	25	Ppep12	2 Day	82
pPep2	1 Day	23	Ppep13	1 Day	28
pPep3	2 Day	80	Ppep14	2 Day	71
pPep4	1 Day	21	Ppep15	1 Day	23
pPep5	1 Day	24	Ppep16	1 Day	24
pPep6	1 Day	27	Ppep17	1 Day	28
pPep7	1 Day	26	Ppep18	1 Day	24
pPep8	1 Day	29	pPep19	1 Day	29
pPep9	1 Day	22	pPep20	1 Day	19
pPep10	1 Day	24	pPep21	1 Day	23

Of the 21 peptides that were tested, the one-day inhibitor peptides inhibited the bacterial growth rate at a level of approximately 25%, while the two-day inhibitor peptides inhibited the bacterial growth rate at levels greater than 75%. As can be seen from the hypothetical data in Table 9, a one-day inhibitor which inhibited the growth rate at 25% would have only reached an OD₅₅₀ of 0.226 when the control strain reached an OD₅₅₀ of 0.64. At that point in time, the growth of the culture that is inhibited by a one-day inhibitor (as measured by the end-point OD₅₅₀) will only be only 35.3% of that of a control strain at that point: thus the inhibitor peptide would have effectively inhibited the growth of the culture by 64.7%. A two-day inhibitor which inhibited the growth rate at 75% would have only reached an OD₅₅₀ of 0.028 when the control strain reached an OD₅₅₀ of 0.64. Thus the growth of the culture that is being inhibited by a two-day inhibitor will only be 4.4% of that of the control strain at this point, and the inhibitor peptide would have effectively inhibited the growth of the culture by 95.6%. These calculations are consistent with the observation that two-day inhibitors prevent the growth of bacteria on plates for a full 48 hours

while the one-day inhibitors only prevent the growth of bacteria on plates for 24 hours.

All 21 candidates were examined using restriction analysis to determine whether they contained 66 bp inserts as expected. While most of them did, the two day inhibitors pPep3 and pPep14 were found to contain a huge deletion. Sequence analysis of these clones revealed that the deletion had caused the carboxy-terminal end of the inhibitor peptides to become fused to the amino-terminal end of the short 63 amino acid Rop protein. The *rop* gene which is part of the ColE1 replicon is located downstream from where the oligonucleotide library is inserted into the pLAC11 vector.

Sequence Analysis of the Top 10 "Anchorless" Inhibitor Peptides. The DNA fragments comprising the sequences encoding the top 10 "anchorless" inhibitor peptides (i.e., excluding the two Rop fusion peptides) were sequenced, and their coding regions are shown in Table 11. Stop codons are represented by stars, and the landmark Bgl II and EcoR I restriction sites for the insert region are underlined. Since the ends of the oligonucleotide from which these inhibitors were constructed contained these restriction sites, the oligonucleotide was not gel isolated when the libraries were prepared in order to maximize the oligonucleotide yields. Because of this, several of the inhibitory clones were found to contain one (n-1) or two (n-2) base deletions in the randomized portion of the oligonucleotide.

Table 11: Sequence analysis of the insert region from the top 10 inhibitory clones and the peptides that they are predicted to encode

5 pPep1 - 13 aa

CAG GAA AGA TTT ATG GTG AAT GAA TTT TTT GGT TTT TTT GAG GAA GTG CCG TAG TAA TAG TGG AAG GGT
M V T E F C D L L F Q I F * * * (SEQ ID NO: 31)

GAA ATT AAT AAG AAT TT (SEQ ID NO: 32)

10

pPep5 - 16 aa

CAG GAA AGA TTT ATG TGG CGG GAG TCA TGG ATT AAG GGT AAG GAG GTG GGG TTT ATG GGT TAA AAT AGT
M W R E S W I K G F L V G F M D * (SEQ ID

15

NO: 36)

TGG ATA ATA AGA AAT T (SEQ ID NO: 37)

pPep6 - 42 aa - last 25 aa could form a hydrophobic membrane-spanning domain

CAG GAA AGA TTT ATG TCA GGG GGA CAT GTG AGG AGG GAG TGC AAG TCG GGG ATG TCC AAT CCG TGG ATC
M S G G H V T E E C K S A M S N E W I

TAC GAA ATA AGA AAT CTC ATG TTT GAG AGC TTA TCA TCG ATA AGC TTT AAT GTG GTA GTT TAT CAC AGT
Y V I E I L M F D S L S S I S F N A V V Y E E

25

TAA (SEQ ID NO: 38)

* (SEQ ID NO: 39)

30 pPep7 - 6 aa

CAG GAA AGA TTT ATG TAT TTG TTC ATC GGA TAA TAC TTA ATG CTC CGG TGG AGA ACT TCA GTT TAA TAA
M Y L F I G * (SEQ ID NO: 40)

GAA TTC (SEQ ID NO: 41)

35

pPep8 - 21 aa

CAG GAA AGA TTT ATG CTT GAA TTT GCG GGG GAG TGG GGG CAG AAA GCG GGA TAC TTT AAT GTG GTA GGG
M L L F G G I C G G K A G Y F T V L F

TCA AGG TAA TAA GAA TTT (SEQ ID NO: 42)

40

S F * * (SEQ ID NO: 43)

pPep10 - 20 aa - predicted to be 45% β -sheet -amino acids 6-14

CAG GAA AGA TTT ATG ATT GGG GGA TGG TTT AGC TTT GCG TGG GCA ATA GTT CAT AAT AAG AAT TTT CAT
M I G G S I C E A W A I V C N F N S F

45

ATT TGA (SEQ ID NO: 44)

V * (SEQ ID NO: 45)

50

pPep12 - 14 aa

CAG GAA AGA TCT ATG AAG GGC GGA AG AAA GAA ATT TGG GAG A A GAA AG TTT TAA AGA GTT AAG AAT
M N A E C E E P I E C E E A A * (SEQ ID NO: 47)
TCT GGT AAT AAG GGT TCT (SEQ ID NO: 47)

5

pPep13 -16 aa - predicted to be 72% α -helical - amino acids
3-15

10 CAG GAA AGA TCT ATG GAG GGT GAA GTG ATG TAT GTG GGA AAA CAG GAA TGG AAG GGA GGA AGG GGA TAT
M D E E V M C A A E L E W F E E T E *
(SEQ ID NO: 48)
GCC GCG TAA TAA GAA TTC (SEQ ID NO: 49)

pPep17 - 12 aa

15 CAG GAA AGA TCT ATG TAT GGT AAT GGA GTG GGA GGA GAT GGT TTA GGT GGA GGC AGC TAT GGA TTT
M * M I C I E A T Y E F
AAT GCA TAA TAA GAA TTC (SEQ ID NO: 50)
M F * * (SEQ ID NO: 51)

20

pPep19 - 5 aa

CAG GAA AGA TCT ATG AGG GGC GCC AAC TAA GGG GGG GGG AAG GTA TTT GTC CGG TGC ATA ATC TCG GGT
M R G A N * (SEQ ID NO: 52)
GTT GTC TAA TAA GAA TTC (SEQ ID NO: 53)

25

Eight out of the top 10 inhibitors were predicted to encode peptides which terminate before the double TAA TAA termination site which was engineered into the oligonucleotide. Two of the inhibitors, pPep6 and pPep10, which contain deletions within the randomized portion of the oligonucleotide, are terminated beyond the EcoR I site. One of the inhibitors, pPep17, contains a termination signal just after the ATG start codon. However, just downstream from this is a Shine Dalgarno site and a GTG codon which should function as the start codon. Interestingly, the start sites of several proteins such as Rop are identical to that proposed for the pPep17 peptide (G. Cesareni et al., Proc. Natl. Acad. Sci. USA. 79:6313-6317 (1982)). The average and median length for the 8 peptides whose termination signals occurred before or at the double TAA TAA termination site was 13 amino acids.

The characteristics of the predicted coding regions of the inhibitor peptides proved to be quite interesting. Three out of the top 10 peptides, pPep1, pPep13, and pPep17, contained a proline residue as their last (C-terminal) amino acid. Additionally, one of the peptides, pPep12, contained 2 proline residues

40

near the C-terminus, at the n-2 and n-3 positions. Thus there appears to be a bias for the placement of proline residues at or near the end of several of the inhibitory peptides. Secondary structure analysis predicted that 3 out of the 10 peptides contained a known motif that could potentially form a very stable
5 structure. pPep13, a peptide containing a C-terminal proline, is predicted to be 72% α -helical, pPep10 is predicted to be 45% β -sheet, and pPep6 is predicted to form a hydrophobic membrane spanning domain.

Verifying that the Inhibitory Clones do not Function as Antisense. To rule
10 out the possibility that the bioactivity of the inhibitory clones resulted from their functioning as antisense RNA or DNA (thus hybridizing to host DNA or RNA) rather than by way of the encoded peptides, the insert regions between the Bgl II and EcoR I sites for the top five inhibitors from Table 10 were recloned into the pLAC11 vector using oligonucleotides which converted the ATG start codon to
15 an ACG codon thus abolishing the start site. In all five cases the new constructs were no longer inhibitory (see Table 12), thus confirming that it is the encoded peptides that causes the inhibition and not the DNA or transcribed mRNA.

Table 12: Antisense test of the top 5 "anchorless" inhibitory peptides from

Inhibitory peptide	% inhibition versus pLAC11 control	Antisense construct	% inhibition versus pLAC11 control
pPep1	26	pPep1-anti	0
pPep5	23	pPep5-anti	0
pPep12	80	pPep12-anti	0
pPep13	28	pPep13-anti	0
pPep19	29	pPep19-anti	0

- 5 Growth rates for cells containing the induced inhibitors or antisense constructs were determined and then the % inhibition was calculated by comparing these values to the growth rate of cells that contained the induced pCyt-3 vector.

DISCUSSION

10

Use of the tightly regulable pLAC11 expression vector made possible the identification of novel bioactive peptides. The bioactive peptides identified using the system described in this Example inhibit the growth of the host organism (*E. coli*) on minimal media. Moreover, bioactive peptides thus identified are, by reason of the selection process itself, stable in the host's cellular environment. Peptides that are unstable in the host cell, whether or not bioactive, will be degraded; those that have short half-lives are, as a result, not part of the selectable pool. The selection system thus makes it possible to identify and characterize novel, stable, degradation-resistant bioactive peptides in essentially a single experiment.

20

The stability of the inhibitory peptides identified in this Example may be related to the presence of certain shared structural features. For example, three out of the top 10 inhibitory "anchorless" (i.e., non-Rop fusion) peptides contained a proline residue as their last amino acid. According to the genetic code, a randomly generated oligonucleotide such as the one used in this Example

25

has only a 6% chance of encoding a proline at a given position, yet the frequency of a C-terminal proline among the top ten inhibitory peptides is a full 30%. This 5-fold bias in favor of a C-terminal proline is quite surprising, because although the presence of proline in a polypeptide chain generally protects biologically

5 active proteins against nonspecific enzymatic degradation, a group of enzymes exists that specifically recognize proline at or near the N- and C-termini of peptide substrates. Indeed, proline-specific peptidases have been discovered that cover practically all situations where a proline residue might occur in a potential substrate (D.F. Cunningham et al., *Biochimica et Biophysica Acta* **1343**:160-186

10 (1997)). For example, although the N-terminal sequences Xaa-Pro-Yaa- and Xaa-Pro-Pro-Yaa (SEQ ID NO: 54) have been identified as being protective against nonspecific N-terminal degradation, the former sequence is cleaved by aminopeptidase P (at the Xaa-Pro bond) and dipeptidyl peptidases IV and II (at the -Pro-Yaa-bond)) (Table 5, G. Vanhoof et al., *FASEB J.* **9**:736-44 (1995);

15 D.F. Cunningham et al., *Biochimica et Biophysica Acta* **1343**:160-186 (1997)); and the latter sequence, present in bradykinin, interleukin 6, factor XII and erythropoietin, is possibly cleaved by consecutive action of aminopeptidase P and dipeptidyl peptidase IV (DPPIV), or by prolyl oligopeptidase (post Pro-Pro bond) (Table 5, G. Vanhoof et al., *FASEB J.* **9**:736-44 (1995)). Prolyl

20 oligopeptidase is also known to cleave Pro-Xaa bonds in peptides that contain an N-terminal acyl-Yaa-Pro-Xaa sequence (D.F. Cunningham et al., *Biochimica et Biophysica Acta* **1343**:160-186 (1997)). Other proline specific peptidases acting on the N-terminus of substrates include prolidase, proline iminopeptidase and prolinase. Prolyl carboxypeptidase and carboxypeptidase P, on the other hand,

25 cleave C-terminal residues from peptides with proline being the preferred P₁ residue (D.F. Cunningham et al., *Biochimica et Biophysica Acta* **1343**:160-186 (1997)).

Also of interest with respect to the stability of the inhibitory peptides, three of the top ten (30%) contained motifs that were predicted, using standard

30 protein structure prediction algorithms, to form stable secondary structures. One of the peptides (which also has a C-terminal proline) was predicted to be 72% α -helical. Another was predicted to be 45% β -sheet; this peptide may dimerize in order to effect the hydrogen bonding necessary to form the β -sheet. A third was

predicted to possess a hydrophobic membrane spanning domain. According to these algorithms (see, e.g., P. Chou et al., *Adv. Enzymol.* **47**:45-148 (1978); J. Garnier et al., *J. Mol. Biol.* **120**:97-120 (1978); P. Chou, "Prediction of protein structural classes from amino acid composition." In *Prediction of Protein Structure and the Principles of Protein Conformation* (Fasman, G.D. ed.), Plenum Press, New York, N.Y. 549-586 (1990); P. Klein et al., *Biochim. Biophys. Acta* **815**:468-476 (1985)), a randomly generated oligonucleotide such as the one used in our studies would have had no better than a 1 in a 1000 chance of generating the motifs that occurred in these peptides.

10 Finally, two of the three two-day inhibitors proved to be fusion peptides in which the carboxyl terminus of the peptides was fused to the amino terminus of the Rop protein. Rop is a small 63 amino acid protein that consists of two antiparallel α -helices connected by a sharp hairpin loop. It is a dispensable part of the ColE1 replicon which is used by plasmids such as pBr322, and it can be
15 deleted without causing any ill-effects on the replication, partitioning, or copy numbers of plasmids that contain a ColE1 ori (X. Soberon, *Gene* **9**: 287-305 (1980). Rop is known to possess a highly stable structure (W. Eberle et al., *Biochem.* **29**:7402-7407 (1990); S. Betz et al., *Biochemistry* **36**:2450-2458 (1997)), and thus it could be serving as a stable protein anchor for these two
20 peptides.

 Table 13 lists naturally occurring bioactive peptides whose structures have been determined. Most of these peptides contain ordered structures, further highlighting the importance of structural stabilization. Research on developing novel synthetic inhibitory peptides for use as potential therapeutic agents over
25 the last few years has shown that peptide stability is a major problem that must be solved if designer synthetic peptides are to become a mainstay in the pharmaceutical industry (J. Bai et al., *Crit. Rev. Ther. Drug.* **12**:339-371 (1995); R. Egleton Peptides. **18**:1431-1439 (1997); L. Wearley, *Crit Rev Ther Drug Carrier Syst.* **8**: 331-394 (1991). The system described in this Example
30 represents a major advance in the art of peptide drug development by biasing the selection process in favor of bioactive peptides that exhibit a high degree of stability in an intracellular environment.

Table 13: Structural motifs observed in naturally occurring bioactive peptides

Bioactive Peptide	Size in Amino acids	Structural Motif	Reference
Dermaseptin	34	α -helix	34
Endorphin	30	α -helix	7
Glucagon	29	α -helix	6
Magainins ^a	23	α -helix	5
Mastoparan	14	α -helix	11
Melittin	26	α -helix	44
Motilin	22	α -helix	25
PKI (5-24)	20	α -helix	38
Secretin	27	α -helix	8
Atrial Natriuretic Peptide	28	disulfide bonds	33
Calcitonin	32	disulfide bonds	4
Conotoxins ^a	10-30	disulfide bonds	37
Defensins ^a	29-34	disulfide bonds	30
EETI II	29	disulfide bonds	23
Oxytocin	9	disulfide bonds	45
Somatostatin	14	disulfide bonds	35
Vasopressin	9	disulfide bonds	20
Bombesin	14	disordered	12
Histatin	24	disordered	51
Substance P	11	disordered	50

^a These peptides belong to multi-member families.

Example III

Directed synthesis of stable synthetically engineered inhibitor peptides

5 These experiments were directed toward increasing the number of
bioactive peptides produced by the selection method described in Example II. In
the initial experiment, randomized peptides fused to the Rop protein, at either the
N- or C- terminus, were evaluated. In the second experiment, nucleic acid
sequences encoding peptides containing a randomized internal amino acid
10 sequence flanked by terminal prolines were evaluated. Other experiments
included engineering into the peptides an α -helical structural motif, and
engineering in a cluster of opposite charges at the N- and C-termini of the
peptide.

15

MATERIALS AND METHODS

Media. Rich LB and minimal M9 media used in this study was prepared as
described by Miller (see Example I). Ampicillin was used in rich media at a
20 final concentration of 100 ug/ml and in minimal media at a final concentration of
50 ug/ml. IPTG was added to media at a final concentration of 1 mM.

Chemicals and Reagents. Extension reactions were carried out using Klenow
from New England Biolabs (Bedford, MA) while ligation reactions were
25 performed using T4 DNA ligase from Life Sciences (Gaithersburg, MD)
Alkaline phosphatase (calf intestinal mucosa) from Pharmacia (Piscataway, NJ)
was used for dephosphorylation. IPTG was obtained from Diagnostic Chemicals
Limited (Oxford, CT).

30 **Bacterial Strains and Plasmids.** ALS225, which is MC1061/*F'lacI^qZ⁺Y⁺A⁺*,
was the *E. coli* bacterial strain used in this study (see Example I). The genotype
for MC1061 is *araD139* Δ (*araABOIC-leu*)*76⁻9* Δ (*lac*)*X74 galU galK rpsL hsr-*
hsm+ as previously described. pLAC11 (Example I), a highly regulable

expression vector, was used to make p-Rop(C) and p(N)Rop- fusion vectors as well as the other randomized peptide libraries which are described below.

Construction of the p-Rop(C) Fusion Vector. The forward primer 5' TAC
5 TAT AGA TCT ATG ACC AAA CAG GAA AAA ACC GCC 3' (SEQ ID NO:
55) and the reverse primer 5' TAT ACG TAT TCA GTT GCT CAC ATG TTC
TTT CCT GCG 3' (SEQ ID NO: 56) were used to PCR amplify a 558 bp DNA
fragment using pBR322 as a template. This fragment contained a Bgl II
restriction site which was incorporated into the forward primer followed by an
10 ATG start codon and the Rop coding region. The fragment extended beyond the
Rop stop codon through the Afl III restriction site in pBR322. The amplified
dsDNA was gel isolated, restricted with Bgl II and Afl III, and then ligated into
the pLAC11 expression vector which had been digested with the same two
restriction enzymes. The resulting p-Rop(C) fusion vector is 2623 bp in size
15 (Fig. 7).

Construction of the p(N)Rop- Fusion Vector. The forward primer 5' AAT
TCA TAC TAT AGA TCT ATG ACC AAA CAG GAA AAA ACC GC 3'
(SEQ ID NO: 57) and the reverse primer 5' TAT ATA ATA CAT GTC AGA
20 ATT CGA GGT TTT CAC CGT CAT CAC 3' (SEQ ID NO: 58) were used to
PCR amplify a 201 bp DNA fragment using pBR322 as a template. This
fragment contained a Bgl II restriction site which was incorporated into the
forward primer followed by an ATG start codon and the Rop coding region. The
reverse primer placed an EcoR I restriction site just before the Rop TGA stop
25 codon and an Afl III restriction site immediately after the Rop TGA stop codon.
The amplified dsDNA was gel isolated, restricted with Bgl II and Afl III, and
then ligated into the pLAC11 expression vector which had been digested with
the same two restriction enzymes. The resulting p(N)Rop- fusion vector is 2262
bp in size (Fig. 8).

30

Generation of Rop Fusion Randomized Peptide Libraries. Peptide
libraries were constructed as described in Example II. The synthetic
oligonucleotide 5' TAC TAT AGA TCT ATG (NNN)₂₀ CAT AGA TCT GCG

TGC TGT GAT 3' (SEQ ID NO: 59) was used to construct the randomized peptide libraries for use with the p-Rop(C) fusion vector, substantially as described in Example II. The complementary strand of this oligonucleotide was generated by a fill-in reaction with Klenow using an equimolar amount of the
5 oligonucleotide primer 5' ATC ACA GCA CGC AGA TCT ATG 3' were used (SEQ ID NO: 60). After extension the resulting dsDNA was digested with Bgl II and ligated into the pLAC11 expression vector which had been digested with the same restriction enzyme and subsequently dephosphorylated using alkaline phosphatase. Because of the way the oligonucleotide library has been
10 engineered, either orientation of the incoming digested double-stranded DNA fragment results in a fusion product.

To construct the randomized peptide libraries for use with the p(N)Rop-fusion vector, the randomized oligonucleotide 5' TAC TAT GAA TTC (NNN)₂₀ GAA TTC TGC CAC CAC TAC TAT 3' (SEQ ID NO: 61), and the primer 5'
15 ATA GTA GTG GTG GCA GAA TTC 3' (SEQ ID NO: 62) were used. After extension the resulting dsDNA was digested with EcoRI and ligated into the pLAC11 expression vector which had been digested with the same restriction enzyme and subsequently dephosphorylated using alkaline phosphatase. Because of the way the oligonucleotide library has been engineered, either orientation of
20 the incoming digested double-stranded DNA fragment results in a fusion product.

Generation of a Randomized Peptide Library Containing Terminal

Prolines. Randomized 20 amino acid peptide libraries containing two proline
25 residues at both the amino and the carboxy terminal ends of the peptides were constructed using the synthetic oligonucleotide 5' TAC TAT AGA TCT ATG CCG CCG (NNN)₁₆ CCG CCG TAA TAA GAA TTC GTA CAT 3' (SEQ ID NO: 63). The complementary strand of the 93 base randomized oligonucleotide was generated by filling-in with Klenow using the oligonucleotide primer 5'
30 ATG TAC GAA TTC TTA TTA CGG CGG 3' (SEQ ID NO: 64). After extension the resulting dsDNA was digested with Bgl II and EcoR I and ligated into the pLAC11 expression vector which had been digested with the same two restriction enzymes. Because the initiating methionine of the peptides coded by

this library is followed by a proline residue, the initiating methionine will be removed (F. Sherman et al. Bioessays 3: 27-31 (1985)). Thus the peptide libraries encoded by this scheme are 20 amino acids in length.

- 5 **Generation of a Randomized Hydrophilic α -Helical Peptide Library.** Table 14 shows the genetic code highlighted to indicate certain amino acid properties.

Table 14: Genetic Code Highlighted to Indicate Amino Acid Properties

TTT phe h_a	TCT ser	TAT <i>tyr</i> b_a	TGT cys
TTC phe h_a	TCC ser	TAC <i>tyr</i> b_a	TGC cys
TTA leu H_a	TCA ser	TAA OCH	TGA OPA
TTG leu H_a	TCG ser	TAG AMB	TGG trp
CTT leu H_a	CCT pro B_a	CAT <i>his</i> h_a	CGT <i>arg</i>
CTC leu H_a	CCC pro B_a	CAC <i>his</i> h_a	CGC <i>arg</i>
CTA leu H_a	CCA pro B_a	CAA <i>gln</i> h_a	CGA <i>arg</i>
CTG leu H_a	CCG pro B_a	CAG <i>gln</i> h_a	CGG <i>arg</i>
ATT ile h_a	ACT thr	AAT <i>asn</i> b_a	AGT ser
ATC ile h_a	ACC thr	AAC <i>asn</i> b_a	AGC ser
ATA ile h_a	ACA thr	AAA <i>lys</i> h_a	AGA <i>arg</i>
ATG met H_a	ACG thr	AAG <i>lys</i> h_a	AGG <i>arg</i>
GTT val h_a	GCT ala H_a	GAT <i>asp</i> h_a	GGT gly B_a
GTC val h_a	GCC ala H_a	GAC <i>asp</i> h_a	GGC gly B_a
GTA val h_a	GCA ala H_a	GAA <i>glu</i> H_a	GGA gly B_a
GTG val h_a	GCG ala H_a	GAG <i>glu</i> H_a	GGG gly B_a

- Boldface** amino acids are hydrophobic while *italicized* amino acids are hydrophilic. The propensity for various amino acids to form α -helical structures is also indicated in this table using the conventions first described by Chou and Fasman (P. Chou et al., *Adv. Enzymol.* **47**:45-148 (1978)). H_a = strong α -helix former, h_a = α -helix former, B_a = strong α -helix breaker, b_a = α -helix breaker. The assignments given in this table are the consensus agreement from several different sources. Hydrophilic versus hydrophobic assignments for the amino acids were made from data found in Wolfenden et. al. (*Biochemistry*, **20**:849-55 (1981));

Miller et. al. (*J. Mol. Biol.* **196**:641-656 (1987)); and Roseman (*J. Mol. Biol.* **200**:513-22(1988)). The propensity for amino acids to form α -helical structures were obtained from consensus agreements of the Chou and Fasman (P. Chou et al., *Adv. Enzymol.* **47**:45-148 (1978); P. Chou, "Prediction of protein structural classes from amino acid compositions," in *Prediction of protein structure and the principles of protein conformation* (G. Fasman, G.D. ed.). Plenum Press, New York, N.Y. 549-586 (1990)); Garnier, Osguthorpe, and Robson (*J. Mol. Biol.* **120**:97-120 (1978)); and O'Neill and DeGrado (*Science*. **250**:646-651 (1990)) methods for predicting secondary structure.

10

By analyzing the distribution pattern of single nucleotides in the genetic code relative to the properties of the amino acids encoded by each nucleotide triplet, a novel synthetic approach was identified that would yield randomized 18 amino acid hydrophilic peptide libraries with a propensity to form α -helices.

15 According to Table 14, the use of a [(CAG)A(TCAG)] codon mixture yields the hydrophilic amino acids His, Gln, Asn, Lys, Asp, and Glu. These amino acids are most often associated with α -helical motifs except for asparagine, which is classified as a weak α -helical breaker. If this codon mixture was used to build an α -helical peptide, asparagine would be expected to occur in about 17% of the

20 positions, which is acceptable in an α -helical structure according to the secondary structure prediction rules of either Chou and Fasman (P. Chou et al., *Adv. Enzymol.* **47**:45-148 (1978); P. Chou, "Prediction of protein structural classes from amino acid compositions," in *Prediction of protein structure and the principles of protein conformation* (G. Fasman, G.D. ed.). Plenum Press, New

25 York, N.Y. 549-586 (1990)) or Garnier, Osguthorpe, and Robson (J. Garnier et al., *J. Mol. Biol.* **120**:97-120 (1978)). Additionally, several well-characterized proteins have been observed to contain up to three α -breaker amino acids within a similarly sized α -helical region of the protein (T. Creighton, "Conformational properties of polypeptide chains," in *Proteins: structures and molecular*

30 *properties*, W.H. Freeman and Company, N.Y., 182-186 (1993)). Since in most α -helices there are 3.6 amino acids per complete turn, the 18 amino acid length was chosen in order to generate α -helical peptides which contained 5 complete turns. Moreover, the use of hydrophilic amino acids would be expected to yield peptides which are soluble in the cellular cytosol.

35

Randomized 18 amino acid hydrophilic α -helical peptide libraries were synthesized using the synthetic oligonucleotide 5' TAC TAT AGA TCT ATG

(VAN)₁₇ TAA TAA GAA TTC TGC CAG CAC TAT 3' (SEQ ID NO: 65). The complementary strand of the 90 base randomized oligonucleotide was generated by filling-in with Klenow using the oligonucleotide primer 5' ATA GTG CTG GCA GAA TTC TTA TTA 3' (SEQ ID NO: 66). After extension the resulting
5 dsDNA was digested with Bgl II and EcoR I and ligated into the pLAC11 expression vector which had been digested with the same two restriction enzymes.

Generating a Randomized Peptide Library Containing the +/- Charge

10 **Ending Motif.** Randomized peptide libraries stabilized by the interaction of oppositely charge amino acids at the amino and carboxy termini were generated according to the scheme shown in Fig. 9. To maximize the potential interactions of the charged amino acids, the larger acidic amino acid glutamate was paired with the smaller basic amino acid lysine, while the smaller acidic amino acid aspartate
15 was paired with the larger basic amino acid arginine. To construct the randomized peptide libraries, the synthetic oligonucleotide 5' TAC TAT AGA TCT ATG GAA GAC GAA GAC (NNN)₁₆ CGT AAA CGT AAA TAA TAA GAA TTC GTA CAT 3' (SEQ ID NO: 67) and the oligonucleotide primer 5' ATG TAC GAA TTC TTA TTA TTT ACG TTT ACG 3' (SEQ ID NO: 68) were used.
20 After extension the resulting dsDNA was digested with Bgl II and EcoR I and ligated into the pLAC11 expression vector which had been digested with the same two restriction enzymes.

For all libraries of randomized oligonucleotides, N denotes that an
25 equimolar mixture of the four nucleotides A, C, G, and T was used, and V denotes that an equimolar mixture of the three nucleotides A, C and G was used. The resulting libraries were transformed into electrocompetent ALS225 *E. coli* cells (Example I) under repressed conditions as described in Example II.

30 **Screening of Transformants to Identify Inhibitor Clones.** Transformants were initially screened using the grid-patching technique to identify any that could not grow on minimal media as described in Example II when the peptides were overproduced. To verify that all the inhibitors were legitimate, plasmid

DNA was made from each inhibitory clone, transformed into a fresh background, then checked to make sure that they were still inhibitory on plates and that their inhibition was dependent on the presence of the inducer, IPTG, as in Example II.

5

Growth Rate Analysis in Liquid Media. Inhibition strength of the peptides was assessed by subjecting the inhibitory clones to a growth rate analysis in liquid media. Minimal or rich cultures containing either the inhibitor to be tested or the relevant vector as a control were diluted to an initial OD₅₅₀ of

10 approximately 0.01 using new media and induced with 1 mM IPTG. OD₅₅₀ readings were then taken hourly until the cultures had passed log phase. Growth rates were determined as the spectrophotometric change in OD₅₅₀ per unit time within the log phase of growth, and inhibition of the growth rate was calculated for the inhibitors using the appropriate vector as a control.

15

RESULTS

Isolation and Characterization of Inhibitor Peptides that are Fused at Their Carboxy Terminal End to the Amino Terminal End of the Rop Protein.

20 Approximately 10,000 peptides protected by the Rop protein at their carboxy terminal end were screened using the grid-patching technique described in Example II, and 16 two day inhibitors were isolated. The inhibitory effects were determined as described in the Example II, using pRop(C) as a control. Unlike the anchorless inhibitors identified in Example II that were only inhibitory on

25 minimal media, many of the Rop fusion inhibitors were also inhibitory on rich media as well, which reflects increased potency. As indicated in Table 15, the inhibitors inhibited the bacterial growth rate at levels that averaged 90% in minimal media and at levels that averaged 50% in rich media. The data in Table 15 is the average of duplicate experiments.

30

Table 15: Inhibitory effects of peptide inhibitors stabilized by fusing the carboxy terminal end of the peptide to the amino terminal end of the Rop protein (Rop(C) fusion peptide inhibitors

Inhibitor	% inhibition in minimal media	% inhibition in rich media
PRop(C)1	87	47
PRop(C)2	99	58
PRop(C)3	85	54
PRop(C)4	98	49
PRop(C)5	95	54
PRop(C)6	99	46
PRop(C)7	91	59
PRop(C)8	86	51
PRop(C)9	93	57
PRop(C)10	91	35

5

Isolation and Characterization of Inhibitor Peptides that are Fused at Their Amino Terminal End to the Carboxy Terminal End of the Rop Protein.

Approximately 6000 peptides protected at their amino terminal end by Rop protein were screened using the grid-patching technique described in Example II. and 14 two day inhibitors were isolated. As observed for the Rop fusion peptides isolated using the p-Rop(C) vector, most of the inhibitor peptides isolated using the p(N)Rop- vector were inhibitory on rich media as well as minimal media. The inhibitors were verified as described hereinabove and subjected to growth rate analysis using p(N)Rop- as a control in order to determine their potency. As indicated in Table 16, the inhibitors inhibited the bacterial growth rate at levels that averaged 90% in minimal media and at levels that averaged 40% in rich media. The data in Table 16 is the average of duplicate experiments.

20

Table 16: Inhibitory effects of peptide inhibitors stabilized by fusing the amino terminal end of the peptide to the carboxy terminal end of the Rop protein (Rop(N) fusion peptide inhibitors)

Inhibitor	% inhibition in minimal media	% inhibition in rich media
pRop(N)1	81	30
pRop(N)2	96	53
pRop(N)3	95	43
pRop(N)4	92	38
pRop(N)5	99	33
pRop(N)6	93	38
pRop(N)7	87	34
pRop(N)8	91	44
pRop(N)9	95	37
pRop(N)10	96	40

5

Isolation and Characterization of Anchorless Inhibitor Peptides Containing Two Prolines at Both Their Amino Terminal and Carboxy Terminal Ends.

Approximately 7500 peptides were screened using the grid-patching technique described in Example II, and 12 two day inhibitors were isolated. As indicated in Table 17, the top ten inhibitors inhibited the bacterial growth rate at levels that averaged 50% in minimal media. The inhibitory effects were determined as described in the text using pLAC11 as a control. The data in Table 17 is the average of duplicate experiments.

15

Table 17: Inhibitory effects of peptide inhibitors stabilized by two proline residues at both the amino and carboxy terminal ends of the peptide

Inhibitor	% inhibition in minimal media
pPro1	50
pPro2	49
pPro3	50
pPro4	59
pPro5	52
pPro6	93
pPro7	54
pPro8	42
pPro9	41
pPro10	42

5 Sequence analysis of the coding regions for the top ten inhibitors is shown in Table 19. The landmark *Bgl* II and *EcoR* I restriction sites for the insert region are underlined, as are the proline residues.

 Since the ends of the oligonucleotide from which these inhibitors were constructed contained *Bgl* II and *EcoR* I restriction sites, the oligonucleotide was
 10 not gel isolated when the libraries were prepared in order to maximize the oligonucleotide yields. Because of this, three of the inhibitory clones, pPro2, pPro5, and pPro6 were found to contain deletions in the randomized portion of the oligonucleotide.

15 **Table 18: Sequence analysis of the insert region from the proline peptides**

pPro1 - 21aa
 20 AGA TGT ATG CCG CCG ATT CTA TGG CGC GAA GCG AIA AAG CGC TTG TGG GGT GGG GAT CAT ACA CCG CCG TAA TAA
 M P I L W G E A F K F L W G G D H T E E * *
 (SEQ ID NO: 70)
 GAA TTT (SEQ ID NO: 69).

pPro2 - 27aa
 25 AGA TGT ATG CCG CCG CCG TTG GAT ATT GTG TCG GGT ATT GAG CTA GCG GGT CAT TTG TGG TGG CGC CGT ATT AAG

M E F F L D I V S G I E V G G F I W C F R I F
AGA TCT CAT GTT TGA (SEQ ID NO: 71)
 N E H V * (SEQ ID NO: 72)

5

pPro3 - 8aa

AGA TCT ATG CCG CCG GAC AAT CCG GTG CTG TGA TGA AGG GGA GTT CGA GGA AGG GGA TAT CAG CCG CCG TAA TAA
 M E E L N F V L * * (SEQ ID NO: 74)

10 GAA TTC (SEQ ID NO: 73)

pPro4 - 9aa

AGA TCT ATG CCG CCG CTA TTG GAG GGA GAT GAG AAA TAG ATA TAT CCG TGG TTG TTT TTC TGT CCG CCG TAA TAA
 M E E L L R G D I K * (SEQ ID NO: 76)

15 GAA TTC (SEQ ID NO: 75)

pPro5 - 10aa

AGA TCT ATG CCG CCG AGG TGG AAG ATG TTG ATA AGA CAG TGA CAG ATG CTT TCC ATT ACT CCG GCG GTA ATA AGA
 M E E R W K M L I R Q * (SEQ ID NO: 78)

20 ATT C (SEQ ID NO: 77)

pPro6 - 7aa

AGA TCT ATG ATG AGA GTA GCG CCG CCG TAA TAA GAA TTC (SEQ ID NO: 79)
 M M R V A E E * * (SEQ ID NO: 80)

25

pPro7 - 14aa

AGA TCT ATG CCG CCG TTG CCG GCG GCA TGC GAT GTA TAT GGG GTA AAT TGA ATG TCT TGT GGG CCG CCG TAA TAA
 M E E L R G A C D V Y G V R * (SEQ ID NO: 82)

30 GAA TTC (SEQ ID NO: 81)

pPro8 - 21aa

AGA TCT ATG CCG CCG GCG AGA GCG GAA CCG GTG GGA GTG ACA TGC TTG AGC GCG AAC GTG TAC CCG CCG TAA TAA
 M E E G R G E A V G V T C L S A N V Y E E * *
 (SEQ ID NO: 84)
GAA TTC (SEQ ID NO: 85)

35

pPro9 - 21aa

AGA TCT ATG CCG CCG GGA AGG GTA GTG TTC TTT GTC GCT ATC TTT GTT TCC GCA ATA TGC CTC CCG CCG TAA TAA
 M P E G R V V F F V A I F V S A I C L P P * *
 (SEQ ID NO: 86)
GAA TTC (SEQ ID NO: 85)

40

pPro10 - 21aa

AGA TCT ATG CCG CCG AGG TTC GGT CAT GAG AGT GTT AAA GCG CTG GCG GAC GTT ACA AAA GCT CCG CCG TAA TAA
 M P E P F A H E S V H G L G D V T K A E E * *
 (SEQ ID NO: 88)
GAA TTC (SEQ ID NO: 87)

45

- 50 All the inhibitors were found to contain two proline residues at either their amino or carboxy termini as expected. Four inhibitors contained two proline residues at both their amino and carboxy termini, five inhibitors contained two proline residues at only their amino termini, and one inhibitor contained two proline residues at only its carboxy terminus.

Isolation and Characterization of Anchorless Hydrophilic Inhibitor

Peptides Stabilized by an α -Helical Motif. Approximately 12,000 peptides were screened using the grid-patching technique and 5 two day inhibitors were isolated.

- 5 The inhibitors were verified as already described for the Rop-peptide fusion studies and subjected to growth rate analysis using pLAC11 as a control in order to determine their potency. As indicated in Table 19, the inhibitor peptides inhibited the bacterial growth rate at levels that averaged 50% in minimal media. The averaged values of two independent determinations are shown.

10

Table 19: Inhibitory effects of the hydrophilic α -helical peptides

Inhibitor	% inhibition in minimal media
pHelix1	67
pHelix2	46
pHelix3	48
pHelix4	45
pHelix5	42

- Sequence analysis of the coding regions for the 5 inhibitors is shown in
- 15 Table 20. The landmark Bgl II and EcoR I restriction sites for the insert region are underlined. Since the ends of the oligonucleotide from which these inhibitors were constructed contained these restriction sites, the oligonucleotide was not gel isolated when the libraries were prepared in order to maximize the oligonucleotide yields. Because of this, two of the inhibitory clones, pHelix2 and pHelix3, were
- 20 found to contain deletions in the randomized portion of the oligonucleotide. The predicted α -helical content of these peptides is indicated in Table 20 according to the secondary structure prediction rules of Garnier, Osguthorpe, and Robson (J. Garnier et al., *J. Mol. Biol.* **120**:97-120 (1978)) prediction rules.

Table 20: Sequence analysis of the insert region from the hydrophilic α -helical peptides

5 pHelix1 - 18aa, 83% α -helical

AGA TCT ATG CAT CAG GAA CAA GAG GAG GAG CAG AAT AAA AAT GAT AAC GAA AAA GAA CAG TAA TAA GAA
M R D E Q E E E H N F F I N E F L R * * (SEQ
ID NO: 90)
TTC (SEQ ID NO: 91)

pHelix2 - 22aa, 68% α -helical

AGA TCT ATG CAG CAG GAG CAG GAG CAA GGC AGG ATG AGC AAG ATG ATG AAG AAT AAT AAG AAT TCT CAT
M Q Q E H E Q G E M C F F N E N N E N F H
GTT TGA (SEQ ID NO: 92)
V * (SEQ ID NO: 93)

pHelix3 - 22aa, 55% α -helical

AGA TCT ATG AAC CAT CAT AAT GAG GGC ATG ATC AAC ACA ATG AAA AGG AGG AAT AAT AAG AAT TCT CAT
M N H E N E A M I E T M E T E N N K N S E
GTT TGA (SEQ ID NO: 93)
V * (SEQ ID NO: 94)

30 pHelix4 - 18aa, 17% α -helical

AGA TCT ATG AAC GAC GAC AAT CAG CAA GAG GAT AAT CAT GAT CAG CAT AAG GAT AAC AAA TAA TAA GAA
M N E D N Q Q E F N E I Q H K D N K * * (SEQ
ID NO: 96)
TTC (SEQ ID NO: 95)

pHelix5 - 18aa, 50% α -helical

AGA TCT ATG CAA GAG CAG GAT CAG CAT AAT GAT AAC CAT CAG GAG GAT AAA CAT AAG AAG TAA TAA GAA
M Q E Q I Q H N I N H H E D E H F K * * (SEQ
ID NO: 98)
TTC (SEQ ID NO: 97)

According to Garnier, Osguthorpe, and Robson secondary structure prediction, all of the encoded peptides are expected to be largely α -helical except for pHelix4. Interestingly, pHelix1 which had the highest degree of α -helical content was also the most potent inhibitory peptide that was isolated in this study.

Isolation and Characterization of Anchorless Inhibitor Peptides Stabilized by an Opposite Charge Ending Motif.

Approximately 20,000 peptides were screened using the grid-patching technique and 6 two day inhibitors were isolated. The inhibitors were verified as already described for the Rop-peptide fusion studies and subjected to growth rate analysis using pLAC11 as a control in order to determine their potency. As indicated in Table 21, the inhibitor peptides inhibited the bacterial growth rate at levels that averaged 50% in minimal media. The averaged values of two independent determinations are shown.

Table 21: Inhibitory effects of peptide inhibitors that are stabilized by the opposite charge ending motif

Inhibitor	% inhibition in minimal media
p+/-1	41
p+/-2	43
p+/-3	48
p+/-4	60
p+/-5	54
p+/-6	85

Sequence analysis of the coding regions for the six inhibitors is shown in Table 22. The landmark Bgl II and EcoR I restriction sites for the insert region are underlined. With the exception of p+/-4 which was terminated prematurely, the coding regions for the inhibitors were as expected based on the motif that was used to generate the peptide libraries.

Table 22: Sequence analysis of the insert region from the opposite charge ending peptides

5 p+/-1 - 25aa

AGA TCT ATG GAA GAC GAA GAC GAG GGT GCG TAA GCG TGG GAA GAA GAA CTT GCG TTS TTS GAG TTS GCG
M E I E I E G A S A W S A E I W C W I S V
CGT AAA CGT AAA TAA TAA GAA TTC (SEQ ID NO: 98)
R K R K * * (SEQ ID NO: 100)

15 p+/-2 - 25aa

AGA TCT ATG GAA GAC GAA GAC GGT CTA GCG ATG GCG GGT GCG TTS GCG AGG CTC ACT TTA TTA TTC TTC
M E I E I G L G M G G R L V E I T L L F F
CGT AAA CGT AAA TAA TAA GAA TTC (SEQ ID NO: 101)
R K R K * * (SEQ ID NO: 102)

20 p+/-3 - 25aa

AGA TCT ATG GAA GAC GAA GAC GGG GAG AGG ATC CAG GCG GCG GGT TGT CTA GTA GCG CTC GTA GAT AAT
M E D E I G E R I Q G A E C P V A L V D R
CGT AAA CGT AAA TAA TAA GAA TTC (SEQ ID NO: 103)
R K R K * * (SEQ ID NO: 104)

30 p+/-4 - 11aa

AGA TCT ATG GAA GAC GAA GAC GAC AGG GCG CGT GCG CGG TAG CTT TAA GTT GCG CTA AGT TSC GAG ATA
M E D E D D R G R G R * (SEQ ID NO: 105)
CGT AAA CGT AAA TAA TAA GAA TTC (SEQ ID NO: 106)

35 p+/-5 - 25aa

AGA TCT ATG GAA GAC GAA GAC GGG GGG GCC GGG AGG AGG GCG TGT CTT TGT TCC GCG CTT GTT GCG GAA
M E D E D G G A G R R A C L C C A L V G E
CGT AAA CGT AAA TAA TAA GAA TTC (SEQ ID NO: 107)
R K R K * * (SEQ ID NO: 108)

40 p+/-6 - 25aa

AGA TCT ATG GAA GAC GAA GAC AAG CGT GCG GAG AGT ACT GCA AAA GAG CGT CAT CTC GGT GCG TCG ATC
M E D E I E R R E R S A K G P E V G F C M
CGT AAA CGT AAA TAA GAC TCT (SEQ ID NO: 109)
R K R K * (SEQ ID NO: 110)

50 DISCUSSION

In Example II, where fully randomized peptides were screened for inhibitory effect, only three peptides (one "anchorless" and two unanticipated Rop fusions resulting from deletion) were identified out of 20,000 potential candidates as a potent (i.e., two day) inhibitor of *E. coli* bacteria. Using a biased

synthesis as in this Example, it was possible to significantly increase the frequency of isolating potent growth inhibitors (see Table 23).

Table 23: Summary of the frequency at which the different types of inhibitor peptides can be isolated

Type of inhibitor peptide	Frequency at which a two day inhibitor peptide can be isolated	Reference
anchorless	1 in 20,000	Example II
protected at the C-terminal end via Rop	1 in 625	This example
protected at the N-terminal end via Rop	1 in 429	This example
protected at both the C-terminal and N-terminal end via two prolines	1 in 625	This example
protected with an α -helix structural motif	1 in 2,400	This example
protected with an opposite charge ending motif	1 in 3,333	This example

Many more aminopeptidases have been identified than carboxypeptidases in both prokaryotic and eukaryotic cells (J. Bai, et al., Pharm. Res. 9:969-978 (1992); J. Brownlees et al., J. Neurochem. 60:793-803 (1993); C. Miller, In *Escherichia coli* and *Salmonella typhimurium* cellular and molecular biology, 2nd edition (Neidhardt, F.C. ed.), ASM Press, Washington, D.C. 1:938-954 (1996)). In the Rop fusion studies, it might have therefore been expected that stabilizing the amino terminal end of the peptide would have been more effective at preventing the action of exopeptidases than stabilizing the carboxy end of the peptides. Surprisingly, it was found that stabilizing either end of the peptide caused about the same effect.

Peptides could also be stabilized by the addition of two proline residues at the amino and/or carboxy termini, the incorporation of opposite charge ending amino acids at the amino and carboxy termini, or the use of helix-generating hydrophilic amino acids. As shown in Table 23, the frequency at which potent inhibitor peptides could be isolated increased significantly over that of the anchorless peptides characterized in Example II.

These findings can be directly implemented to design more effective peptide drugs that are resistant to degradation by peptidases. In this example, several strategies were shown to stabilize peptides in a bacterial host. Because the aminopeptidases and carboxypeptidases that have been characterized in

5 prokaryotic and eukaryotic systems appear to function quite similarly (C. Miller, In *Escherichia coli* and *Salmonella typhimurium* cellular and molecular biology, 2nd edition (Neidhardt, F.C. ed.), ASM Press, Washington, D.C. 1:938-954 (1996); N. Rawlings et al., *Biochem J.* 290:205-218 (1993)), the incorporation of

10 on or more of these motifs into new or known peptide drugs should slow or prevent the action of exopeptidases in a eukaryotic host cell as well.

Sequence Listing Free Text

SEQ ID NO:2

15 peptide sequence having opposite charge ending motif

SEQ ID NOs:3-4

stabilized angiotensin

20 SEQ ID NOs:6-19, 24-28, 55-58, 60, 62, 64, 66, 68

primer

SEQ ID NOs:20-22

primer fragment

25

SEQ ID NOs:23, 59, 61, 63, 65, 67

randomized oligonucleotide

SEQ ID NOs:29-33

30 antisense oligonucleotide

SEQ ID NOs:34, 36, 39, 40, 43, 45, 46, 48, 51, 52, 70, 72, 74, 76, 78, 80, 82, 84,
86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 105, 108, 110
stabilized peptide

- 5 SEQ ID NOs:35, 37, 38, 41, 42, 44, 47, 49, 50, 53, 69, 71, 73, 75, 77, 79, 81, 83,
85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 106, 107, 109
nucleic acid encoding stabilized peptide

SEQ ID NO:54

- 10 N-terminal protective sequence

The foregoing detailed description and examples have been given for
clarity of understanding only. No unnecessary limitations are to be understood
therefrom. The invention is not limited to the exact details shown and described,

- 15 for variations obvious to one skilled in the art will be included within the
invention defined by the claim.